Pak. J. Bot., 51(3): 855-864, 2019. DOI: 10.30848/PJB2019-3(36)

OVER-EXPRESSED *HSP 17.6B*, ENCODING HSP20-LIKE CHAPERONES SUPERFAMILY PROTEIN, CONFERS HEAT STRESS TOLERANCE IN *ARABIDOPSIS THALIANA*

FAIZA TAWAB[1], IQBAL MUNIR[1*], FARHATULLAH[2] AND AQIB IQBAL[1]

[1]*Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar Pakistan*
[2] *Department of Plant Breeding and genetics, The University of Agriculture, Peshawar Pakistan*
[*]*Corresponding author's email: iqmunir@hotmail.com; Tele: +92-91-9221072; Fax: +92-91-9221072*

Abstract

Abiotic stresses are the key hazard limitation to plant development and sustainable agriculture throughout the world. The investigation of stress tolerant genes by genome scale transcriptome analysis will provide opportunities for the development of stress tolerant crop varieties. Here, we report a potential stress tolerance gene *HSP17.6B* (*AT2G29500*) in *Arabidopsis thaliana* that can confer tolerance to *Arabidopsis* plants upon overexpression. *HSP17.6B* overexpression resulted in higher root elongation, increased plants survival rate, reduced electrolyte leakage and retention of chlorophyll contents under heat stress condition in comparison to wild-type plants. Overall, we showed *AtHSP17.6B* as a potential heat stress tolerance candidate in *Arabidopsis thaliana*.

Key words: Arabidopsis, RNA-seq analysis, Heat stress tolerance.

Introduction

Owing to their sessile lifestyle, plants are continuously affected by a complex array of environmental stresses which require their rapid and proper response for adaptation and survival. Plants grown under tropical and subtropical climatic conditions are highly affected by the increase in temperature and therefore become a key preventive aspect for field crop production (Wahid *et al.,* 2007). Different genetic approaches can be used to develop thermo-tolerant crop plants to mitigate the adverse effects of heat stress. In this rationale it is imperative to comprehensively understand the physiological responses, different mechanisms of plants to high temperature and also the potential strategies for improving crop thermo-tolerance.

Plants have evolved several molecular mechanisms for adaptation and subsequent survival under stress conditions as revealed by stress induced transcriptomics. At molecular level the plant responses to heat stress by the expression of a diverse group of heat shock proteins, other proteins related to stress and also the reactive oxygen species (ROS) production. In totality all these mechanisms which are operated at molecular level allow the plants to flourish under high temperature stress.

The response of heat stress in a broad range of organisms has been well renowned. Increase in temperature results in the assembly of specific families of proteins recognized as heat shock proteins (HSPs) (Howarth & Ougham, 1993). On the basis of their molecular mass, HSPs have been classified into different families, the majority of which are chaperon in function (Jaenicke & Creighton, 1993). The major families of HSPs are HSP90s, HSP70s and small HSPs from which all organisms produce HSPs; however the plants are unique in the number of different small HSPs that they produce (Jakob & Buchner, 1994). Majority of the studies conducted to investigate the heat stress responses in plants have focused on HSPs (Schoffl *et al*., 1999). However, the possible role of heat shock proteins in stress tolerance including small heat-shock proteins (smHSPs) is not fully explored.

The HSP20 proteins are actually molecular chaperones that require no ATP and usually form oligomeric protein complexes of 200-800 kDa, ranging from 9 to 50 subunits. These protein complexes prevent protein denaturation in both eukaryotic and prokaryotic cells by acting on them (Cashikar *et al*., 2005). These chaperones can also assist other chaperones in helping to maintain the native conformation of nascent polypeptide chains and in reorganizing denatured proteins to their native conformation. The HSP20 proteins main characteristic is their highly conserved (80-100 amino acids) sequence referred to as the alpha crystallin domain (ACD) that is positioned in the C-terminal region of the protein. The ACD domain is further divided into two regions, N-terminal consensus I (127 amino acids) and C-terminal consensus II (29 amino acids) that is estranged by variable length hydrophobic region (Scharf *et al*., 2001). In Arabidopsis 19 genes encodes Hsp20, based on their subcellular localization and homology they are grouped into 12 subfamilies (Alavilli *et al*., 2016).

Arabidopsis thaliana is used globally as a model plant for basic research due to its small genome, easy to handle, short life cycle and easy to be genetically manipulated. The gene function of *A. thaliana* provides the fundamental source to formulate the hypotheses and to design experiments concerning other economically significant plants species. Therefore the fundamental research and practical applications require in depth perceptive of the *A. thaliana* genome and a comprehensive and precise understanding of the expression of its related genes. The development of innovative genetic and genomic assets and novel procedures of data attainment as well as analysis can help achieve this goal (Klepikova *et al.,* 2016).

Genomic sequencing provides an opportunity to discover a large number of stress related pathways and genes that can serve up as the basis for crop improvement. For instance, stress related changes in genome transcripts can be investigated by next-generation sequencing (NGS) technology coupled with high-throughput transcriptome profiling (Molina *et al*., 2011). RNA-seq is more economical and high throughput compared with whole-

genome sequencing, therefore it is becoming an important part of functional genome research (Alsford *et al*., 2011). The detailed information about the gene expression at the mRNA level is obtained by the transcriptomic analysis and is therefore extensively used to monitor the stress responsive candidate genes.

In this study we aimed to utilize the publicly available transcriptome data of plants exposed to heat stress in order to identify potential stress tolerance gene that can be employed in plant improvement programs to enable plants combat the adverse growth conditions. We identified At-HSP17.6B gene, belonging to cytoplasmic class 17.6 kDa small heat shock protein (HSP20) family, as a potential target for heat stress tolerance in *Arabidopsis thaliana.*

Material and Methods

RNA-seq public datasets retrieval and analysis using tuxedo protocol: To gain insights of transcriptome wide genes expression, different publically available RNA-seq data sets (SRA 009031 and GSE72806 of Filichkin *et al*., (2010) and Suzuki *et al*., (2016), respectively were retrieved from National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) and European Molecular Biology Laboratory Nucleotide Archive (EMBL-ENA) in Sequence Read Archives (SRA) format and were converted to FASTQ format using SRA toolkit "NCBI SRA toolkit" for downstream analysis using Tuxedo Protocol (Trapnell *et al*., 2012). RNA-seq analysis was done using 'TOPHAT-Cufflinks' pipeline. Data visualization of the Differentially Expressed Genes (DEGs) was done using CummeRbund, and it also used other R packages to construct expression graphs (for single gene) and heat maps (for a group of genes) under heat, drought and salinity. Gene ontology analysis was done to determine the biological functions of the differentially expressed genes using GENECODIS (Carmona-Saez *et al*., 2007). This enabled us to categorize the miss regulated genes into broad categories based on the similarities in their functions. GO-enrichment analysis was performed on the differentially expressed genes to get insights on their biological functions (Biological Processes; BP), molecular functions (Molecular Functions; MF), cellular compartment (Cellular Compartment; CC) and metabolic pathways. Common target genes of different stresses were determined using Venny2 (available at: bioinfogp.cnb.csic.es/tools/venny). Gene investigator was used to visualize the transcript level expression of *HSP17.6B* gene in different tissues and cell types under normal growth conditions.

Plant material, growth conditions and stress treatment: For all experiments Columbia accession (Col-0) of *Arabidopsis thaliana* was used. Arabidopsis seeds were first sterilized by treating with 70% ethanol twice for 1 min, then seeds were sterilized using 50 % bleach/0.1% Tween 20 for 1 min and finally rinsed thrice in sterilized water. After sterilization seeds were grown on Petri dishes with 15 cm diameter containing about 20 mL of MS agar media (Murashige & Skoog, 1962) supplemented with 1.5% sucrose. All plants were grown under different environmental conditions and exposed to heat stresses according to the growth conditions mentioned in the original studies (Table 3).

Expression analysis: For transcript level expression of genes and validation of RNA-seq data, RNA was extracted from *Arabidopsis thaliana* plants using TRI Reagent® (Sigma-aldrich), and cDNA was synthesized from 2 µg total RNA using SuperscriptII reverse transcriptase (Invitrogen, Carlsbad, CA). A two-step real-time PCR reaction was performed using Light Cycler® 480 instrument. The expression was checked in three technical replicates that contained 100 ng of cDNA with two biological replicates. For confirmation of *HSP17.6B* mRNA level overexpression, semi quantitative PCR was used while using Ubiquitin gene as a loading control. Whereas western blot was used for protein level expression confirmation of overexpression lines. For this purpose protein was isolated from the wild-type (Col-0) and overexpression lines and western blotting was performed to check the expression level of our gene at protein level using anti- HA monoclonal antibody. Ponceau staining was used for loading equal amount of proteins.

Generation of over expression lines: The potential multi-stress tolerance *HSP 17.6B* gene was amplified with gene specific primers (Supplementary Table 2) and cloned into a binary vector containing a 35S Cauliflower Mosaic fused Virus (CaMV) promoter. *Arabidopsis thaliana* plants at flowering stage were subjected to *Agrobacterium* mediated gene transfer procedure at primary inflorescences stage in sucrose containing growth media. The dipping of the plants inflorescences in solution was done only for 30 seconds and then enclosed in polythene bags for 24 hours for the conservancy of moisture. The dipping treatment was carried out twice within 14 days. The seeds were collected at maturity.

Measurement of physiological and biochemical indexes: To access the functionality of *HSP17.6B* as a potential heat stress tolerance candidate, performance of the over-expressed transgenic lines was determined in comparison with wild-type plants. Differences in the fresh weight of wild-type and over-expressed transgenic plants was determined. Survival rate (%) was determined by subjecting both the wild-type and over-expressed transgenic lines to heat stress of 45^{o}C for 1 hr and then kept at room temperature followed by calculating the alive plants percentage. Relative Electrolyte Leakage (REL) was measured by conductivity detector DDS-11A (Kangyi) to determine the conductivity. Chlorophyll content of leaves was measured spectrophotometrically (U-2810, Hitachi) by the method described by Arnon, 1949. Briefly, plant leaf samples (control and stressed) were harvested and kept for 4 weeks. Leaf samples (~ 100 mg) was homogenized in 3 ml of 80% acetone and left for 30 min in dark to extract the chlorophyll and supernatant was collected by centrifugation. Chlorophyll a and b were determined in totality using the following formula; (12.7 x A_{663} - 2.69 x A_{646}) x Volume / Weight = Chl *a* mg/g FW and (22.9 x A_{646} - 4.86 x A_{663}) x Volume / Weight = Chl *b* mg/g FW. Relative root elongation (%) was measured as the percentage of root elongation in heat stress condition normalized over control condition.

Table 1. Expression of the shortlisted heat stress responsive genes in multi-stress conditions with their FPKM values.

S. No.	Genes Ids	Stress condition	FPKM values
1.	AT2G29500	Drought	134.011
		Heat	19686.9
		Salt	90.9127
		Control	2.91449
2.	AT4G12400	Drought	40.2849
		Heat	4887.22
		Salt	233.2595
		Control	2.31271
3.	AT4G10250	Drought	25.3183
		Heat	5147.24
		Salt	18.595
		Control	2.44858
4	AT5G52640	Drought	68.4057
		Heat	6558.34
		Salt	47.722
		Control	5.09076
5.	AT3G12580	Drought	112.229
		Heat	7532.12
		Salt	90.5156
		Control	7.28461
6.	AT2G32120	Drought	8.63293
		Heat	1434.7
		Salt	5.93672
		Control	1.72381
7.	AT5G48570	Drought	22.5811
		Heat	3084.79
		Salt	15.7793
		Control	5.35733
8.	AT2G20560	Drought	25.7095
		Heat	2653.76
		Salt	23.5585
		Control	5.33787
9.	AT5G59720	Drought	83.3641
		Heat	14544.6
		Salt	57.2587
		Control	39.0914
10.	AT1G07350	Drought	36.9816
		Heat	1473.97
		Salt	24.6202
		Control	7.93949
11.	AT5G64510	Drought	6.76311
		Heat	229.324
		Salt	5.58397
		Control	1.78264
12.	AT1G07400	Drought	142.172
		Heat	23536.3
		Salt	132.253
		Control	21.0999

Results

Global gene expression analysis and functional classification of genes in response to heat stress: In this study, our basic understanding of the plant acclimation to abiotic stresses was extended through whole transcriptome (RNA-Seq) analysis based on the utilization of the publicly available transcriptome data sets of plants exposed to heat stress conditions. Based on our RNA seq analysis SRA009031 (Filichkin *et al*., 2010) and GSE72806 (Suzuki *et al*., 2016) a large number of genes were differentially expressed under heat stress condition. Further, to determine their functions, all the DEGs genes in response to heat stress in the selected data sets were combined for the GO (Gene ontology) analysis. The highly enriched GO terms at biological process (BP) were 'translation activity' and 'response to salt stress' in case of up-regulated genes. While 'biosynthesis of secondary metabolites', 'plant pathogen interaction' and 'starch and sucrose metabolism' were highly enriched GO terms with down-regulated genes. In case of molecular function (MF), the highly enriched GO terms were 'protein binding', 'DNA binding' and 'catalytic activity'. Whereas, the cellular compartment (CC) gene ontology analyses showed 'cytosol', 'chloroplast' and 'plasma membrane' as the highly enriched compartments with DEGs (Differentially Expressed Genes) under heat stress condition. Additionally, DEGs induced by heat stress were enriched in 'biosynthesis of secondary metabolites' followed by 'ribosome' (Fig. 6).

Short-listing of heat stress responsive genes: All the up-regulated genes from the selected data sets were used as an input for the construction of Venn diagram using Venny2 (available at: bioinfogp.cnb.csic.es/tools/venny). A total of six hundred and eighty two genes were commonly up-regulated in response to heat stress (Fig. 1A). Further, a total of 12 genes were short-listed based on highest fold change and lowest q value (Table 1). These genes included *HSP17.6B, HOP3, HSP22.0, HSP90-1, HSP17.8, MED37C, HSP70-8, FKBP65, AT2G20560, HSP18.1, SR45A* and *TIN1*. We then calculated the transcript abundance for all shortlisted genes in response to drought, heat and salt stresses using the selected RNA-seq datasets, SRA009031 (Filichkin *et al*., 2010) and GSE72806 (Suzuki *et al*., 2016). Our analysis showed all shortlisted genes to be highly induced under heat stress compared to other abiotic stresses (Table 1). As the *HSP17.6B* (*AT2G29500*) showed highest expression amongst all shortlisted genes (Fig. 1B, Table 1) and not much is known about its functions, therefore, we proceeded with *HSP17.6B* for further experiments.

Expression pattern of *HSP 17.6B*: To assess the expression patterns of *HSP17.6B* in different cell and tissue types, Gene investigator (https://genevestigator.com/gv/) was used to investigate the mRNA expression levels of *HSP17.6B* in public database derived from different tissues (Fig. 1E). The highest level of *HSP17.6B* mRNA expression was found in the 'sperm cells', 'guard cells protoplast' followed by 'leaf protoplast' and 'root phloem pole pericycle protoplast', this suggests the possible role of *HSP17.6B* in tissue types and organelles.

To validate the mRNA expression of *HSP17.6B* under different abiotic stresses, quantitative PCR analyses were performed on the seedling grown under drought (25% PEG, Three-wk-old), heat (43^{o}C for 1hr, Three-wk-old) and salt (200 mM NaCl for 2hrs, two week-old)) stresses as previously mentioned in their respective publications (Filichkin *et al*., 2010 and Suzuki *et al*., 2016). The RNA-seq analysis showed a dramatic induction in the transcript levels of *HSP17.6B* under heat stress as compared to other abiotic stresses (Fig. 1C). This was further validated by our qPCR results for *HSP17.6B* transcript expression as it showed consistent patterns with the RNA-seq results (Fig. 1D). mRNA levels of *HSP17.6B* were up-regulated by ~180 folds under heat stress (Fig. 1D), about 10 folds in salt stress (Fig. 1D), while the mRNA levels were increased by ~8 folds in the drought stress (Fig. 1D). Consistency in the expression pattern of qPCR and RNA-seq data highlights the reliability of RNA-Seq data.

Table 2. Size and primer detail of selected gene for generation of over expression lines.

Selected genes in heat stress condition	Size of gDNA	Primers detail
AT2G29500	1.05 kb	AGTGGAAAAGGAAGGTGGCT FP TGTTGAAGCGAGTGTGTTTTG RP

Table 3. Highlighting the growth and abiotic stress conditions that were used in this study with their original references and also the GEO accession number for the respective public RNA-seq database.

	Plant material	Growth conditions	Abiotic stress	Reference	GEO/SRA number
1.	Col-0	16:8h day/night	Heat Stress (42°C) Three-wk-old	Filichkin *et al*., 2010	SRA009031
2.	Col-0	12h/12 (L/D)	Heat stress (25days old) 06:00–09:00, 21°C; 09:00–17:00, 43°C; 17:00–09:00, 21°C	Suzuki *et al*., 2016	GSE72806
3.	Col-0	21C, 12h (L/D)	44°C 1,2,3hrs	Suzuki *et al*., 2016	GSE72806

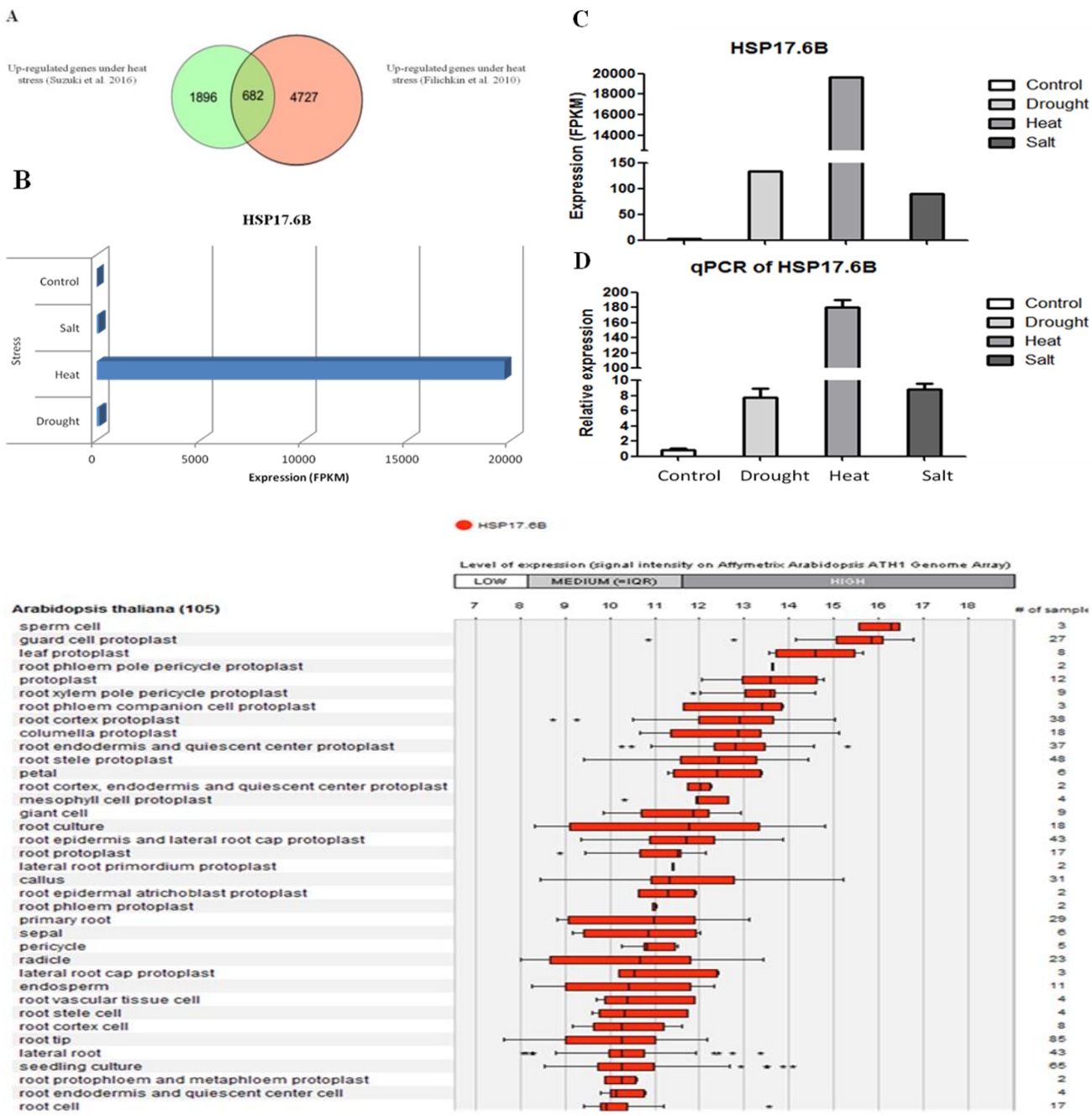

Fig. 1. (A) Venn diagram representation of the number of up-regulated genes for heat stress tolerance that are unique and common in the selected data sets of Suzuki *et al.,* 2016 and Filichkin *et al*., 2010. (B) Expression (FPKM) of *HSP17.6B* gene in salt, heat and drought stress in comparison with control condition. (C) & (D) RNA-seq and Quantitative PCR analysis of the selected *HSP17.6B* gene under different abiotic stresses. Each bar represents the mean expression of two biological and three technical replicates. (E) Expression pattern of selected gene *HSP 17.6B* across different tissues and cell types using Gene Investigator.

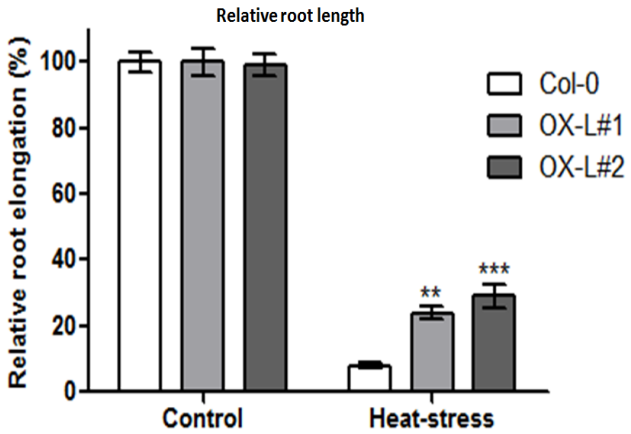

Fig. 2. Effect of heat stress on root elongation of *Arabidopsis* seedlings. The primary root lengths of wild-type and overexpression transgenic lines were measured when 4 days seedlings were subjected to heat stress of 45°C for 1 hr. Data are presented as the relative root elongation (RRE) compared to the control values. Each column is significantly different at $P < 0.05$ under heat stress condition.

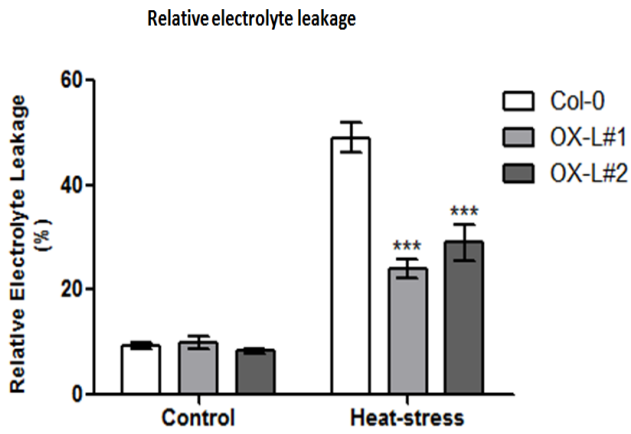

Fig. 4. Relative electrolyte leakage (%) of wild-type and overexpression transgenic lines under control and heat stress condition (45°C, 1hr). Data are presented as the relative electrolyte leakage (REL) compared to the control values. The overexpression lines are significantly different at $p<0.05$ compared to control condition.

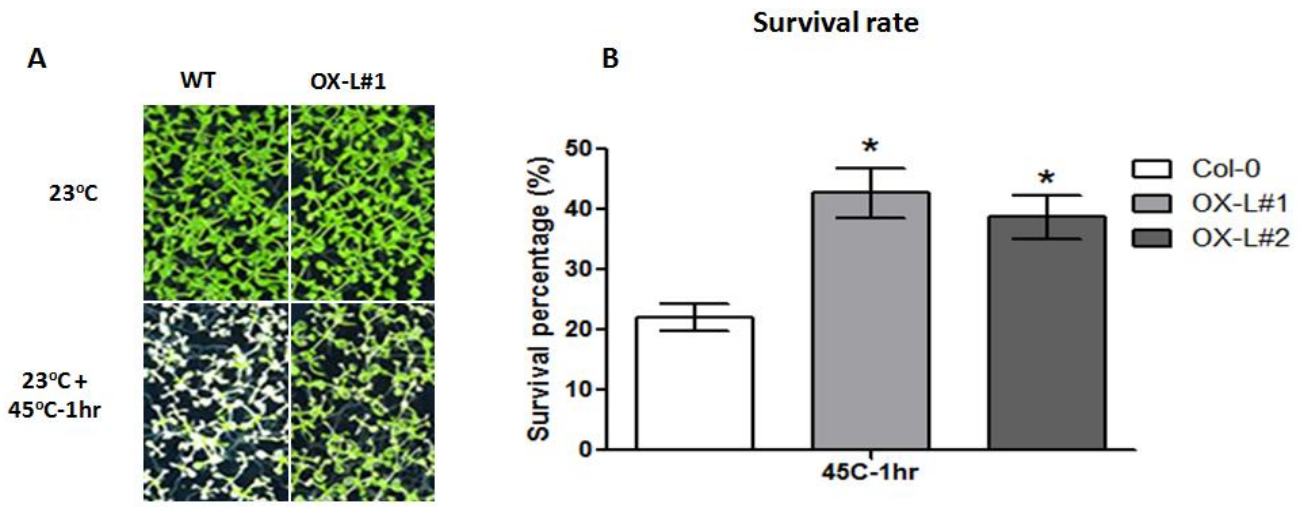

Fig. 3. A. Phenotypic differences in wild-type and overexpression lines after the completion of heat stress (45°C, 1hr). B. Effect of heat stress on the survival rate (%) of the overexpression and wild-type plants. The overexpression lines are significantly different at $p<0.05$ compared to control condition.

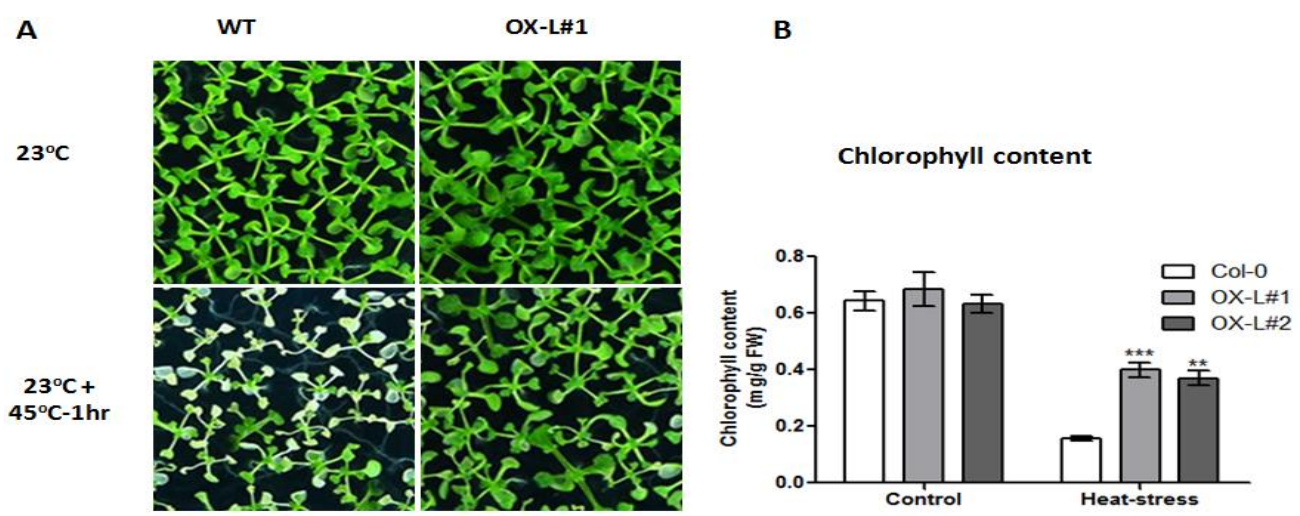

Fig. 5. A. Phenotypic differences in wild-type and overexpression lines after the completion of heat stress (45°C, 1hr), B. Chlorophyll content (mg/g FW) of wild-type and overexpression transgenic lines under control and heat stress conditions. The overexpression lines are significantly different at $P < 0.05$ compared to control condition.

SUPPLEMENTARY DATA

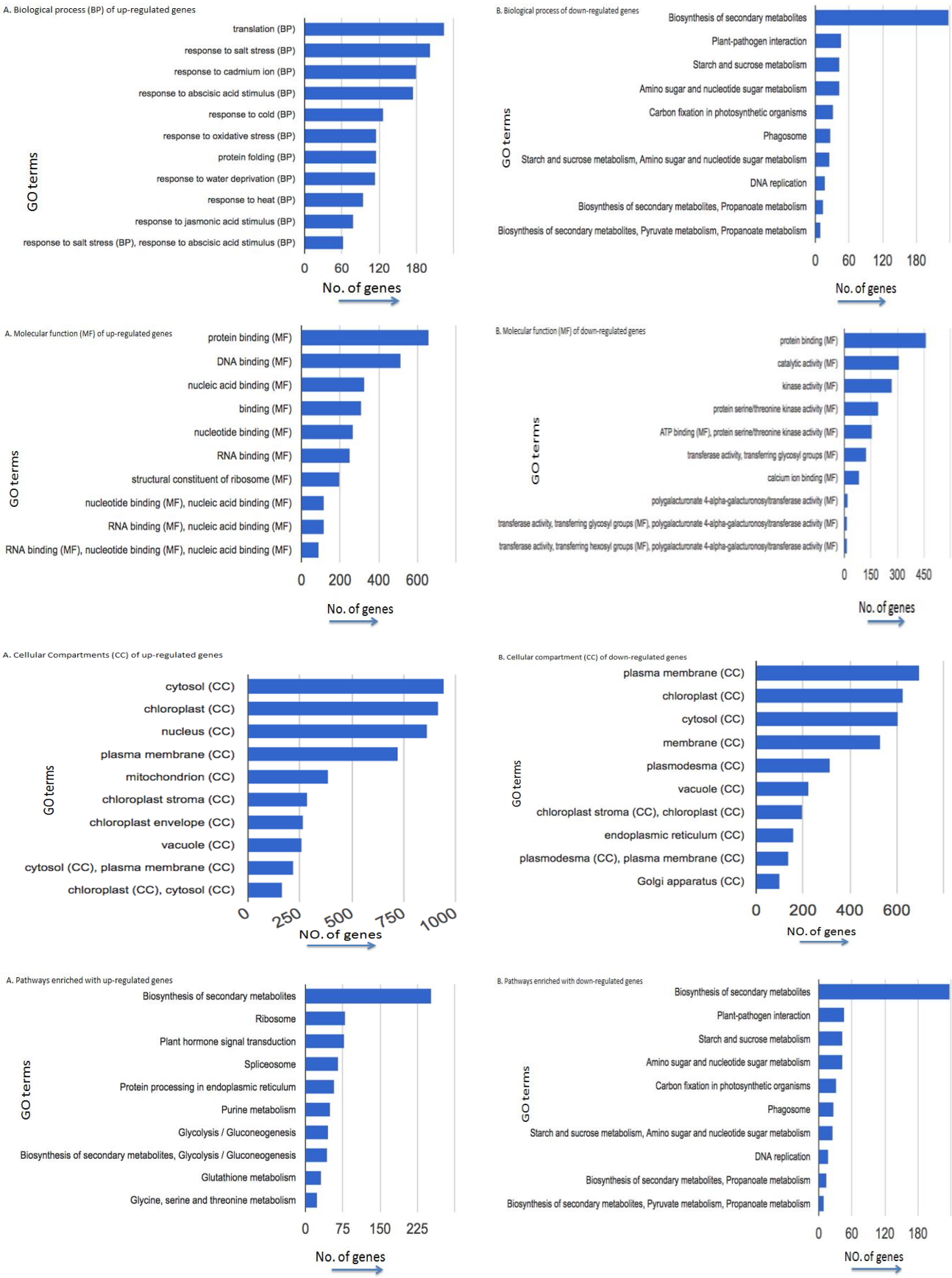

Fig. 6. Functional classification of heat stress induced up/down-regulated genes (Flichiken *et al*., 2010 and Suzuki *et al*., 2016) for Biological Process (BP), molecular Functions (MF), Cellular Compartments (MF) and Metabolic Pathways using a threshold P -value of 0.05. A. Up-regulated genes B. Down regulated genes.

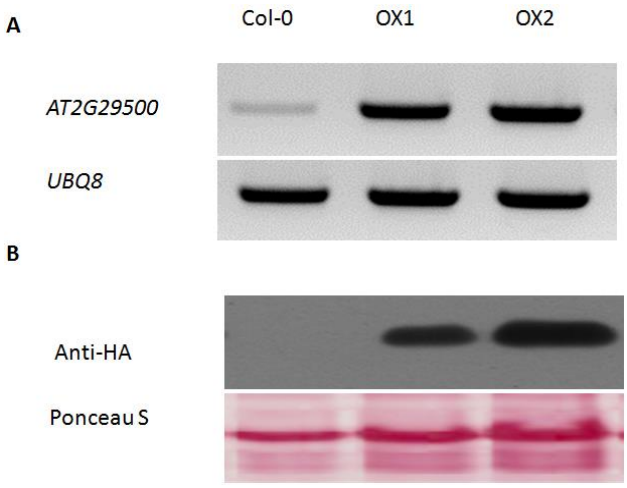

Fig. 7. (A) Gel picture of over-expressed gene *HSP17.6B* of RT-PCR to confirm its overexpression at transcript level, *UBQ8* was used a loading control (B) Protein level confirmation was done by western blot using anti- HA monoclonal antibody, Ponceau staining (Ponceau S) was used as loading control for loading equal amount of protein.

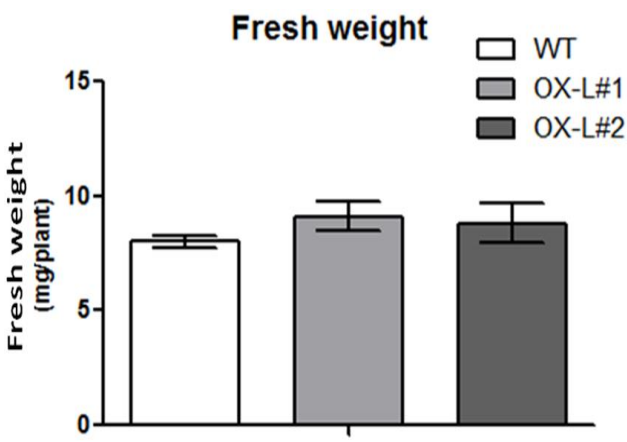

Fig. 8. Fresh weight (mg/plant) of wild-type and *HSP17.6B* overexpression lines grown under normal growth conditions. The overexpression plants shows wild-type like phenotype under normal growth condition.

Cloning of *HSP 17.6B*: For generation of overexpression lines, the potential heat tolerance gene *HSP17.6B* gene was fused with 3X-HA tag and cloned into *pCHF3* vector, harboring a CaMV 35S promoter using specific primers (Table 3). The *HSP17.6B* over-expressed transgenic lines were confirmed by RT-PCR at transcript level as the expression was significantly higher than wild-type plants (Fig. 7A). To confirm if the introduced *HSP17.6B* transcripts are translated into proteins, the protein level expression was measured through western blot. We were able to detect the recombinant protein in the over-expressed lines only using anti-HA monoclonal antibody (Fig. 7B).

Phenotypic and physiological response of the over-expressed transgenic lines with *HSP17.6B* gene: The over-expressed transgenic lines were first observed for their fresh weight under normal growth conditions. However, we did not observed any significant differences in the fresh weight of over-expressed transgenic lines and wild-type control plants (Fig. 8). As reduced root length is one of the effects of heat stress on plants (Wahid *et al*., 2007), relative root lengths were recorded for the *HSP17.6B* overexpression transgenic lines compared to wild-type control plants. Significantly higher root lengths were observed under heat stress condition as compared to the wild-type plants (Fig. 2). Heat stresses are often lethal to the plants and hence wild-type plants often show low survival rates. To know if the *HSP17.6B* overexpression can rescue the low survival rates, survival rates were recorded for both the overexpression transgenic lines and wild-type plants after exposure to a heat stress of 45°C for 1 hour. Our results showed that the overexpression lines showed a two-fold higher survival rate of ~40 % compared to wild-types plants with a survival rate of around 20% (Fig. 3).

Different biotic and abiotic stresses affect various aspects of plant physiology and cellular structures, one of the common phenomenon associated with these stresses is the electrolyte leakage (Demidchik *et al*., 2014). Like other stresses, heat stress also induces electrolyte leakage in plants, often resulting in cell death (Liu & Huang, 2000). In order to determine if the overexpression lines are resistant to the lethal level of electrolyte leakage, we measured the electrolyte leakage under heat stress (45^{o}C for 1 hr) for both overexpression lines and wild-type plants. Under heat stress, significantly lower relative electrolyte leakage was recorded for the overexpression transgenic lines than the wild-type plants. Precisely, about two folds reduced leakage was found in the overexpression lines as compared to the wild-type plants (Fig. 4) whereas, under control conditions, all of the genotypes showed almost equal relative electrolyte leakage. Chlorophylls are essential components of the photosynthetic apparatus of plants, the power generation machinery. Heat stress induces degradation of chlorophyll in the leaf tissues causing leaf senescence (Jespersen *et al*., 2016). To assess the effect of *HSP17.6B* overexpression on chlorophyll content, we measured the chlorophyll content in overexpression and wild-type plants under heat stress and control conditions. Significantly higher chlorophyll content were observed in the overexpression transgenic lines as compared to the wild-type plants, precisely, ~2.2 fold higher chlorophyll contents were recorded for overexpression lines (Fig. 5), whereas, both overexpression and wild-type plants showed similar chlorophyll content under control conditions (without heat stress) (Fig. 5). Taken together, our findings suggests that the overexpression of *HSP17.6B* makes the plant more tolerant to heat stress by improving different physiological parameters of the plants i.e., by reducing the negative effect of heat on plant roots elongation, enabling plants to absorb more water and combat heat by the cooling effect of transpiration, secondly by preventing electrolyte leakage from plant cells and retention of comparatively higher chlorophyll contents.

Discussion

Being sessile in nature the plants are continuously exposed to diverse environmental stresses. Extensive research in model plants and crops has aimed to understand the plant responses to a range of biotic and abiotic stresses, as these stresses reduce harvest yields (Hirayama & Shinozaki, 2010). In response to abiotic stresses, hundreds

of plant genes are differentially regulated as demonstrated by RNA-seq analyses. In this study, our basic understanding of the plant acclimation to abiotic stresses was extended through whole transcriptome (RNA-Seq) analysis based on the utilization of the publicly available transcriptome data sets of plants exposed to heat stress conditions. Based on our RNA seq analysis of Filichkin *et al*., (2010), we detected a total of 12,606 DEGs (Differentially Expressed Genes) under heat stress, of which 7145 genes were up-regulated while 5461 were down regulated. In the data sets of Suzuki *et al*., (2016), 7543 DEGs were detected in totality, out of which 4092 genes were up regulated and 3451 genes were down regulated. The functional annotation of the differentially expressed genes (DEGs) performed by GO analysis (GENECODIS) categorized these genes into Biological functions (Biological Processes; BP), Molecular functions (Molecular Functions; MF), Cellular compartment (Cellular Compartment; CC) and Pathways effected based on their functions.

The genes up-regulated under heat stress were involved in different biological processes. Overall, the expression analyses suggest that upon exposure to heat stress, the plants increases the expression of genes related to translation machinery for the rapid expression of stress-tolerance genes. Additionally, the heat stress induces expression of genes that are normally up-regulated in salt stress response underlying the common mechanisms for stress responsiveness to heat and salt. A similar inter-stresses-connection among abiotic stresses like salinity, drought, intense temperatures and oxidative stress has been reported earlier by Wang *et al.,* (2003). Further heat stress up-regulated the genes expression involved in oxidative stress and protein folding which is consistent with the findings of Liu and Howel (2010) who stated that the plants earliest metabolic response upon exposure to abiotic stresses was the increase in protein folding and processing. Rasul *et al*., (2017) also highlighted similar phenomenon that under stress condition the plants alter their physiological and biochemical pathways to ensure their survival. For example salt stress induces different pathways that are intended to detoxify the ROS to maintain the ion-homeostasis. Similarly the induction of metabolic pathways takes place during heat stress and the plant thermo-tolerance is governed by the heat shock proteins. The expression analysis for the down regulated genes suggests that upon exposure to eminent level of thermal stress the production of secondary metabolites is highly affected as reported previously by Sacharay *et al*., (2002) who stated that the concentration of anthocynines (a secondary metabolite) are decreased in buds and fruits at elevated temperature. High temperature stress causes the negative regulation of genes concerned in sucrose to starch conversion. These are transporter genes such as HvSUT1 and HvSTP3, the mechanism suggests that stress causes the repression of phloem unloading of sugars (Mangelsena *et al.,* 2011). Same findings were also reported by Zemanek and Frecer (1990) who stated that during heat stress the accumulation of sucrose decreases significantly in winter wheat (*Triticum astivum* L.). Similarly the concentration of starch was recorded to be significantly reduced in heat-stressed leaves of potato (Lafta & Lorenzen, 1995) and Indian mustard (Subrahmanyam & Rathore, 1995). Further, the analysis suggested that as the temperature increases plants photosynthetic capacity is also largely effected and so carbohydrate metabolism as reported previously by Huang *et al*., (1998); Prange *et al*., (1990) and Wolf *et al*., (1991), who stated that high temperature causes the reduction in carbohydrate accumulation that may result from the imbalance between respiration and photosynthesis. Different molecular processes were enriched with up-regulated genes under heat stress. Over all, the analysis suggests that when Arabidopsis is exposed to heat stress it responds by up regulation of genes for protein binding, DNA binding and nucleic acid binding suggesting that binding activity operate predominantly at transcript level (Liu & Howel, 2010; Tang *et al.,* 2013). This might be the fact that the accumulation of heat shock protein that are governed by heat shock factors with DNA binding domains might be the important machinery in the transduction pathway between elevated temperature stress and gene expression (Nover *et al*., 2001) for increased thermo-tolerance. The expression analysis of down regulated genes under heat stress suggests that heat stress affects the genes expression concerned in transcriptional regulation and other metabolic processes. A number of different cellular components were enriched with the up-regulated genes under heat stress. In our study we found that cytosol, chloroplast and nucleus were the highly enriched GO terms. Liu *et al*., (2015) proposed the chloroplasts as heat sensors, because the retrograde signals are generated by the protein translation capacity of chloroplast for the activation of HsfA2-dependent heat-responsive genes in the nucleus. Similarly the temperature variation is perceived by the plasma membrane and then the signal is transduced into the nucleus as a result transcriptome is transformed (Saidi *et al*., 2009; Conde *et al.,* 2011). Over all, the GO terms for the down regulated genes suggests that heat stress negatively affect different cellular structures and membranes, which is consistent with the findings of (Ruelland & Zachowski, 2010) who stated that the stability of proteins, several membranes, RNA structures and cytoskeleton are differently effected by heat stress. The change in photosynthesis is related to the major modifications that occur at the sub-cellular level in the chloroplasts. For instance elevated temperature results in the decrease rate of photosynthesis by altering the structural organization of thylakoid membrane (Karim *et al.,* 1997). A large number of pathways were enriched with up-regulated genes under heat stress. The GO analysis showed that the production of secondary metabolites occur during heat stress, which is consistent with the previous findings that there is an increase in the biosynthesis of phenolics and reduction in their oxidation under heat stress, which is considered to provide heat stress tolerance such as in watermelon (Rivero *et al*., 2001). Heat stress also causes the alteration in hormonal homeostasis, content, biosynthesis, stability and compartmentalization (Maestri *et al.,* 2002). Our analysis showed that the protein processing in endoplasmic reticulum is highly enriched with up-regulated genes. As plants expresses stress proteins to adapt and cope with environmental stresses. Similar results have been reported that plants increase the production of HSPs when rapid or steady increase in temperature occur

(Nakamoto & Hiyama, 1999; Schoffl *et al.,* 1999). Over all the expression analysis of down regulated genes under heat stress suggests that the production of secondary metabolites decreases after thermal treatment as previously reported by Morison & Lawlor (1999) who stated the level of β-carotene decreased when heat stress was applied in *Brassicaceae.* Heat stress down regulated the genes involved in plant pathogen interaction which means that abiotic stresses cause the robust modulation of plants tolerance or susceptibility toward pathogen. This is done by different mechanisms in plants to respond to various biotic and abiotic stresses that direct plant pathogen interactions (Pandey *et al*., 2015). Our analysis showed that thermal stress causes the down regulation of pathways related to amino sugar and nucleotide sugar metabolism, which is consistent with the previous findings that heat stress negatively affects carbon metabolism as well as the levels of specific sugars (Pandey *et al*., 2015). The RNA-seq predicted expression of the selected genes was confirmed by qRT-PCR which showed that the results of both are significantly comparable to each other, and RNA-Seq results are reliable and can be used for further analysis.

The selected multi-stress tolerant gene was over-expressed in *Arabidopsis thaliana*, and its expression pattern was confirmed at transcript and protein level (Fig. 7). The overexpression transgenic *Arabidopsis* lines with *HSP 17.6B* functional attributes were studied to confirm their role in stress condition. Significant differences in the *HSP17.6B* overexpression transgenic lines and wild-type plants were observed under heat stress. Under normal growth condition fresh weight (mg/plant) was determined in which the overexpression transgenic lines showed wild-type like phenotype. Significant differences were noted for the root lengths among the wild-type and over-expressed transgenic lines when 4 days seedlings were exposed to heat stress (45^0C for 1 hr). The overexpression transgenic lines maintained to grow even in heat stress condition as compared to the wild-type plants suggesting that they are more tolerant to heat stress. The survival rate (%) of both the overexpression transgenic lines (OX-L1 & OX-L2) was significantly higher, 45% and 40% respectively, than the wild-type plants which showed only (20%) survival rate under heat stress condition. Significantly increased survival rate in overexpression lines means they are more tolerant to heat stress. It has been documented that high temperature causes the alteration of the physical state of the membrane which lead to fluidization and its breakdown (Los & Murata, 2004). As a result of this disruption the plasma membrane's boundary perturbs and ultimately results in the amplified permeability and ion leakage, which can be calculated readily by the efflux of electrolytes (Wahid *et al.*, 2007). Comparatively lower relative electrolyte leakage was determined for all of the overexpression transgenic lines in comparison with control (Col-0) plants grown under heat stress. This suggests that overexpression lines are significantly more tolerant to heat stress and are capable of stabilizing their cell membrane and hence lower electrolyte leakage under stress condition as compared to control plants. High temperature stress causes a decrease in chlorophyll content (Kozowska, 2007). Significantly higher chlorophyll content was recorded for the over expressed transgenic Arabidopsis plants grown under heat stress condition as compared to wild-type plants suggesting they are more tolerant to heat stress as they are able to stabilize higher chlorophyll content under stress condition. Taken together, our findings suggests that the overexpression of *HSP17.6B* makes the plant more tolerant to heat stress by improving different physiological parameters of the plants i.e. by reducing the negative effect of heat on plant roots elongation, enabling plants to absorb more water and combat heat by the cooling effect of transpiration, secondly by preventing electrolyte leakage from plant cells and retention of comparatively higher chlorophyll contents.

Conclusions and Recommendations

As small heat shock proteins have a molecular chaperone activity which could account for a protective effect at high temperature, *HSP17.6B* was over-expressed in *Arabidopsis thaliana* to study the effect of heat stress on the transgenic plants. *HSP17.6B* (*AT2G29500*) overexpression increased tolerance to heat stress as confirmed by the survival rate, relative root elongation and chlorophyll content of the overexpression lines as compared to wild-type plants under heat stress conditions. Manipulating the orthologues of this gene in economical important crops can be helpful to ensure the global food security in context of global warming. It is recommended to determine the molecular chaperone activity of *HSP17.6B* and the influence of *HSP17.6B* overexpression on signalling pathways (abscisic acid, salicylic acid and ethylene) and reactive oxygen metabolism (catalase, ascorbic acid or glutathione production).

References

Alavilli, H., J.P. Awasthi, G.R. Rout, L. Sahoo, B.H. Lee and S.K. Panda. 2016. Overexpression of a barley aquaporin gene, HvPIP2 confers salt and osmotic stress tolerance in yeast and plants. *Front. Plant Sci.,* 7: 1566.

Alsford, S., D.J. Turner and S.O. Obado. 2011. High-throughput phenotyping using parallel sequencing of RNA interference targets in the African trypanosome. *Genome Res.,* 21(6): 915-924.

Arnon, D.I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris. Plant Physiol*., 24(1): 1-15.

Carmona-Saez, P., M. Chagoyen, F. Tirado, J.M. Carazo and A. Pascual-Montano. 2007. GENECODIS: a web-based tool for finding significant concurrent annotations in gene lists. *Gen. Biol.*, 8(1): R3.

Cashikar, A.G., M. Duennwald and S.L. Lindquist. 2005. A chaperone pathway in protein disaggregation: Hsp26 alters the nature of protein aggregates to facilitate reactivation by Hsp104. *J. Biol. Chem*., 280: 23869-23875.

Conde, A., M.M. Chaves and H. Gerós. 2011. Membrane transport, sensing and signaling in plant adaptation to environmental stress. *Plant Cell Physiol.*, 52: 1583-602.

Demidchik, V., D. Straltsova, S.S. Medvedev, G.A. Pozhvanov, A. Sokolik and V. Yurin. 2014. Stress-induced electrolyte leakage: the role of K+-permeable channels and involvement in programmed cell death and metabolic adjustment. *J. Exp. Bot*., 65(5): 1259-1270.

Filichkin, S.A., D. Henry, A.G. Scott, S. Rongkun, W.B. Douglas, E.F. Samuel, K.W. Wang and C.M. Todd. 2010. Alternative splicing in *Arabidopsis thaliana*. *Genome Res.*, 20: 45-58.

Hirayama, T. and K. Shinozaki. 2007. Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA. *Trends Plant Sci*., 12(8): 343-351.

Howarth, C.J. and H.J. Ougham. 1993. Gene expression under temperature stress. *New Phytol.,* 125: 1-26.

Huang, B., X. Liu and J.D. Fry. 1998. Shoot physiological responses of two bentgrass cultivars to high temperature and poor soil aeration. *Crop Sci.,* 38: 1219-1244.

Jaenicke, R. and T.E. Creighton. 1993. Junior chaperones. *Curr. Biol.,* 3: 234-235.

Jakob, U. and J. Buchner. 1994. Assisting spontaneity: the role of HSP90 and smHSPs as molecular chaperones. *Trends Biochem. Sci.,* 19: 205-211.

Jespersen, D., J. Zhang and B. Huang. 2016. Chlorophyll loss associated with heat-induced senescence in Bentgrass. *J. Plant Sci.,* 249: 1-12.

Karim, M.A., Y. Fracheboud and P. Stamp. 1997. Heat tolerance of maize with reference of some physiological characteristics. *Ann. Bang. Agri.,* 7: 27-33.

Klepikova, A.V., A.S. Kasianov, E.S. Gerasimov, M.D. Logacheva and A.A. Penin. 2016. A high resolution map of the *Arabidopsis thaliana* developmental transcriptome based on RNA-seq profiling. *Plant. J*., 88(6): 1058-1070.

Kozowska, M. 2007. Plant Physiology. From theory to applied sciences (in Polish). PWRiL, Warsaw, Poland.

Lafta, A.M. and J.H. Lorenzen. 1995. Effect of high temperature on plant growth and carbohydrate metabolism in potato. *Plant Physiol.,* 109: 637-643.

Liu, J.X. and S.H. Howel. 2010. Endoplasmic reticulum protein quality control and its relationship to environmental stress responses in plants. *Plant Cell*., 22(9): 2930-2942.

Liu, J., L. Feng, J. Li and Z. He. 2015. Genetic and epigenetic control of plant heat responses. *Front. Plant Sci.,* 06: 1-21.

Liu, X. and B. Huang. 2000. Heat stress injury in relation to membrane lipid peroxidation in creeping Bentgrass. *Crop Sci*., 40: 503-510.

Los, D.A. and N. Murata. 2004. Membrane fluidity and its roles in the perception of environmental signals. *Biochimica et Biophysica Acta,* 1666: 142-157.

Maestri, E., N. Klueva, C. Perrotta, M. Gulli, H.T. Nguyen and N. Marmiroli. 2002. Molecular genetics of heat tolerance and heat shock proteins in cereals. *Plant Mol. Biol.,* 48: 667-681.

Mangelsena, E., J. Kilianb, K. Harterb, C. Janssonc, D. Wankeb and E. Sundberga. 2011. Transcriptome analysis of high-temperature stress in developing barley caryopses: Early stress responses and effects on storage compound biosynthesis. *Mol. Plant,* 4(1): 97-115.

Molina, C., M. Zaman-Allah, F. Khan, N. Fatnassi, R. Horres, B. Rotter, D. Steinhauer, L. Amenc, J.J. Drevon, P. Winter and G. Kahl. 2011. The salt-responsive transcriptome of chickpea roots and nodules via deep super SAGE. *BMC Plant Biol.,* 10(11): 1471-2229.

Morison, J.I.L. and D.W. Lawlor. 1999. Interactions between increasing CO2 concentration and temperature on plant growth. *Plant Cell Environ*., 22: 659-82.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.,* 15(3): 473-497.

Nakamoto, H. and T. Hiyama. 1999. Heat-shock proteins and temperature stress. In: (Ed.): Pessarakli, M. *Handbook of Plant and Crop Stress.* Marcel Dekker, New York, 399-416.

Nover, L., K. Bharti, P. Döring, S.K. Mishra, A. Ganguli and K-D. Scharf. 2001. Arabidopsis and the heat stress transcription factor world: How many heat stress transcription factors do we need? *Cell Stress Chaper.,* 6(3): 177-189.

Pandey, P., V. Ramegowda and M. Senthil-Kumar. 2015. Shared and unique responses of plants to multiple individual stresses and stress combinations: Physiological and molecular mechanisms. *Front. Plant Sci.,* 6: 723.

Prange, R.K., K.B. McRae, D.J. Midmore and R. Deng. 1990. Reduction in potato growth at high temperature: Role of photosynthesis and dark respiration. *Amer. Potato J.,* 67: 357-369.

Rasul, I., H. Nadeem, M.H. Siddique, R.M. Atif, M.A. Ali, A. Umer, F. Rashid, M. Afzal, M. Abid and F. Azeem. 2017. Plants sensory-response mechanisms for salinity and heat stress. *J. Anim. Plant. Sci.,* 27(2): 490-502.

Rivero, R.M., J.M. Ruiz, P.C. García, L.R. López-Lefebre, E. Sánchez and L. Romero. 2001. Resistance to cold and heat stress: Accumulation of phenolic compounds in tomato and watermelon plants. *Plant Sci*., 160(2): 315-321.

Ruelland, E. and A. Zachowski. 2010. How plants sense temperature. *Environ. Exp. Bot.,* 69: 225-232.

Sachray, L., D .Weiss, M. Reuveni, A. Nissim-Levi and M.O. Shamir. 2002. Increased anthocyanin accumulation in aster flowers at elevated temperatures due to magnesium treatment. *Physiol. Plant,* 114: 559-565.

Saidi, Y., A. Finka, M. Muriset, Z. Bromberg, Y.G.Weiss, F.J.M. Maathuis and P. Goloubinoff. 2009. The heat shock response in moss plants is regulated by specific calcium-permeable channels in the plasma membrane. *Plant Cell*., 21: 2829-2843.

Scharf, K.D., M. Siddique and E. Vierling. 2001. The expanding family of Arabidopsis thaliana small heat stress proteins and a new family of proteins containing alpha-crystallin domains (Acd proteins). *Cell Stress & Chaper.,* 6: 225-237.

Schoffl, F., R. Prandl and A. Reindl. 1999. Molecular responses to heat stress. In: (Eds.): Shinozaki, K. & K.Yamaguchi-Shinozaki.. Molecular Responses to Cold, Drought, Heat and Salt Stress in Higher Plants. *R.G. Landes Co., Austin, Texas.* 81-98.

Subrahmanyam, D. and V. S. Rathore. 1995. High temperature influences 14 CO_2 assimilation and allocation of 14C into different biochemical fractions in the leaves of Indian mustard. *J. Agron. Crop Sci.,* 169: 169-175.

Suzuki, N., E. Bassil, J.S. Hamilton, M.A. Inupakutika, S.I. Zandalinas, D. Tripathy, Y. Luo, E. Dion, G. Fukui and A. Kumazaki. 2016. ABA is required for plant acclimation to a combination of salt and heat stress. *Plos One,* 11: 0147625.

Tang, S., H.Y. Liang, D.H. Yan, Y. Zhao, X. Han and J.E. Carlson. 2013. *Populus euphratica*: the transcriptomic response to drought stress. *Plant Mol. Biol.,* 83: 539-557.

Trapnell, C., A. Roberts, L. Goff, G. Pertea, D. Kim, D.R. Kelley, H. Pimentel, S.L. Rinn, J.L. Salzberg and L Pachter. 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Prot.,* 7(3): 562-78.

Wahid, A., S. Gelani, M. Ashraf and M.R. Foolad. 2007. Heat tolerance in plants: an overview. *Environ. Exp. Bot.,* 61: 199-223.

Wang, W., B. Vinocur and A. Altman. 2003. Plant responses to drought, salinity and extreme temperatures: Towards genetic engineering for stress tolerance. *Planta*, 218: 1-14.

Wolf, S., A. Marani and J. Rudich. 1991. Effect of high temperature on carbohydrate metabolism in potato plants. *J. Exp. Bot.,* 42: 619-625.

Zemanek, M. and R. Frecer. 1990. The influence of high temperatures on saccharose accumulation in the grain of winter wheat genotypes. *Rostlinna Vyroba,* 36: 965-976.

(Received for publication 4 April 2018)