Summary & Conclusions
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Heat stress in wheat is known to cause an array of physiological, biochemical and morphological changes which affect its growth and development. Since a decade, heat stress has caused a significant decrease in wheat yield which has made India struggle to match the record production figures, thus posing a critical challenge in maintaining food security. Despite the importance of wheat as a significant cereal crop, current information of its genome sequence is not sufficient for functional genomics. Sequencing of wheat genome is quite a challenge because of its large genome size (16,000 Mb) but is already underway. However, mapping and characterizing ESTs offers a manageable approach to the complex architecture and functioning of the wheat transcriptome and helps in unravelling the genetics of the stress response. Hence, functional genomics of heat stress in wheat was initiated to understand the transcriptional profile of the sensitive and tolerant cultivars of wheat under heat stress by subtractive hybridization, both at the seedling and grain filling stages (Chauhan et al. 2011). Four subtractive cDNA libraries were constructed from three different developmental stages (developing seed, unfertilized flower and seedling). Heat stress associated gene transcripts were identified based on their putative functions and validated by cDNA macroarray and northern/RT-PCR analysis, with the aim to unravel the complexity associated with heat stress response in wheat. To understand the regulation of heat stress tolerance, detailed information about these ESTs and their functional annotation is necessary. Therefore expression profiles were analysed under different different hormones/elicitors and various developmental and growth stages. Based on either their expression profiles or presence at specific developmental stages, some genes were chosen in the present study for detailed functional characterization. Some of the salient features of these genes is as follows:

Functional characterization of TaHSP26 promoter

Previous work in the lab demonstrated expression of HSP26 in different tissues, representing various growth stages of the wheat plant indicating this gene to be highly inducible in unfertilized flower tissues, in both anther and ovary after two hour of heat stress. Its characterization by over- and under-expression in Arabidopsis was also undertaken (Chauhan et al. 2012). Promoter activity of TaHSP26, (P26) was analyzed by quantitative and semi-quantitative RT-PCR and GUS histochemical assay in three-
week-old transgenic *Arabidopsis* plants. To analyze the heat responsiveness of the promoter monocots, rice transgenics were raised and analysed at T2 stage. Histochemical analysis under non-stressed and heat-stressed conditions showed that GUS activity conferred by *TaHSP26* promoter increased progressively with an increase in the HS duration in rice transgenics and was not visible under control conditions. The GUS transcripts in the transgenic lines accumulated as early as 10 mins subsequent to temperature stress thus demonstrating its high responsiveness to heat stress. Histochemical analysis performed on reproductive organs i.e. flower bud, anther, ovary, seed and vegetative organs i.e. seedlings, mature spikes, young spikelets showed intense GUS staining under heat stress conferring stage-specific expression during heat stress. Quantitative RT-PCR for GUS transcript and fluorometric estimation of GUS protein validates the presence of three types of HSEs and other heat stress responsive elements in the close proximity in the promoter. The importance of *TaHSP26* promoter lies in the result where it is highly inducible in reproductive organs such as flower, anther and ovary since flower tissues are more sensitive to stress. Therefore, usefulness of *TaHSP26* promoter for transgene expression in crop plants under heat stress conditions has been proposed.

To identify the cis-regulatory elements present in the *TaHSP26* promoter region responsible for its heat-stress responsiveness, a series of deletions were made by designing primers that truncate promoter fragments. Firstly, the CCAAT box-elements were removed gradually from the promoter in different deletion fragments. Secondly, in order to determine the importance of 5’ Untranslated Region (UTR), this region was deleted in additional constructs. Transgenic *Arabidopsis* plants harbouring full-length promoter of *TaHSP26* gene, when analysed histochemically, revealed a blue colored end product in the heat-stressed transgenics exclusively and activity in control plants. As CCAAT box-elements were gradually removed from the promoter, it resulted in further reduction of GUS gene expression relative to full-length promoter. This highlights the importance of CCAAT-box elements in *TaHSP26* promoter. GUS staining was observed in transgenic plants harbouring full length promoter without UTR as compared to transgenic plants harbouring full-length promoter with UTR, thus establishing the importance of 5’ UTR in *TaHSP26* promoter. GUS activity at the transcriptional level and post-transcriptional level was analysed by quantitative RT-PCR and fluorometric analysis further validating our
results. Interestingly, when HSE alone was present in the promoter fragment and no CCAAT-box elements were present in the deletion fragment, no GUS activity was found. Thus, we conclude that it is the synergistic combination of HSE along with CCAAT box element that is required for TaHSP26 promoter to demonstrate its heat stress inducibility.

**Functional Characterization of TaMIPS2**

Another gene that was studied in detail is TaMIPS, a heat stress inducible enzyme. TaMIPS is expressed during different stages of seed development upon heat stress and molecular evidence indicates that MIPS is crucial during heat stress recovery and flower development (Chauhan et al. 2011c). MIPS gene family was identified in rice and Arabidopsis along with their alternatively spliced forms. Real-time PCR expression analysis was undertaken to decipher the function of rice and Arabidopsis MIPS genes and the spliced variants in 28 different tissues representing major plant growth stages and under different abiotic stresses. Phylogenetic analysis revealed little diversity among various plants MIPS, however, TaMIPS2, cloned in the present investigation, grouped with monocots, while previously reported TaMIPS1, grouped with Arabidopsis. For the subcellular localization of TaMIPS protein, fluorescence confocal microscopy revealed its localization in the plasma membrane as well as nuclear membrane. TaMIPS2 overexpressing Arabidopsis transgenics also demonstrated higher myo-inositol levels under heat stress conditions as compared to wild-type plants, implying its role in possibly mediating heat stress response in plants. Further confirmation of its role in various abiotic stresses has been demonstrated by phenotypical, morphological and biochemical analysis. Thus, TaMP could possibly mediate an ameliorating role in salinity, and cold stress via the ABA pathway. Its role in heat stress has also been well established by several heat tolerance assays.

**Functional Characterization of TaMP**

For functional analysis of TaMP protein, its domain analysis revealed that it belonged to Pmp3 superfamily. It functions to modulate the membrane potential, particularly in resisting high cellular cation concentrations. To unravel the exact location of TaMP on bread wheat homologous chromosomes, PLANTBLAST analysis tool in NCBI was used. Its localization was predicted on all the 3 homeologous chromosomes viz. 5A, 5B and 5D, while PCR from nullisomic lines confirmed its presence on
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chromosome 5A and 5B. The predicted hydrophobic protein TaMP was confirmed to have two transmembrane regions, thus this was further confirmed by protoplast transient expression assay of transgenics. The localization was confirmed in the chloroplasts as examined by confocal microscopy. Arabidopsis transgenics overexpressing TaMP gene showed that transgenics fared better than the wild-type in some of the abiotic stresses. Transgenic seedlings were bigger in size, rosette diameter was greater and root length was more than double the length of wild-type. Morphometric analysis and physiological characterization also showed more tolerance to abiotic stresses in transgenics. Therefore, its possible role in drought and salinity stress is speculated via hormonal signaling.

Functional characterization of TaHSF

Based on sequence homology and domain architecture, TaHsf was categorised under class A HSFs since it has a AHA motif. TaHsfA2d from developing wheat seeds was cloned and characterized. The complete ORF of TaHsfA2d was cloned in pGBKTK7 vector with GAL4 DBD fusion and was shown to be capable of transactivation. Deletion analysis revealed that the activation domain of TaHsfA2d is localized in the region containing the AHA motif. Overexpression of TaHsfA2d was undertaken in Arabidopsis and transgenics were shown to have increased basal thermotolerance. The TahsfA2d overexpressing plants showed better biomass accumulation and faster growth as measured by fresh weight and number of rosette leaves under moderate heat stress environment. The mutant KO hsfa2 plants were also analysed and showed very poor growth and produced almost half the yield as compared to WT plants and quarter yield when compared to overexpression plants. Overexpression of TaHsfA2d has also been shown to have a positive effect on seed yield and silique length and number under moderate heat stress conditions. It also provides salt tolerance in transgenic Arabidopsis. Complimentation of the KO plants with wheat TaHsfA2d, was undertaken and complimented plants performed better then KO plants and displayed almost at par growth with the wild-type plants. Further, the expression analysis of 21 target genes was undertaken under control and heat stress conditions to gain an insight in the molecular basis of TaHsfA2d mediated stress tolerance. It was concluded that heat and salt stress tolerance of the transgenic Arabidopsis plants overexpressing TaHsfA2d is provided by constitutive expression of these stress related genes.
Functional Characterization of TaZnF and TabZIP

Through northern analysis and semi-quantitative RT-PCR, TaZnF has been reported to be highly expressed during high temperatures. It was found to contain a RING domain and a transmembrane domain which is found to have an important role in cellular ubiquitination pathway. Multiple alignments of the full length ZnF protein showed a high sequence similarity of 90% with a Brachypodium uncharacterized protein. Phylogenetic analysis revealed that the C3HC4 zinc fingers are broadly classified into three separate clades and TaZnF belongs to clade III. Expression analysis was undertaken in different tissues representing major plant growth stages including those from different abiotic stresses. TaZnF was found to be expressed largely in roots and its expression was upregulated under most of the abiotic stresses such as BR, SA, PEG, ABA, NaCl, cold and heat stress. The subcellular localization of TaZnF protein showed it to be localized in nucleus as well as plasma membrane. TaZnF overexpression transgenics showed no difference in the phenotype under heat stress. TaZnF does not function as a transcriptional activator as revealed by yeast-two-hybrid.

As revealed by domain analysis, bZIP transcription factors are distinct from other transcription factors due to the presence of a basic region and a leucine zipper in their DNA binding domains. Phylogenetic analysis revealed that bZIP zinc fingers are broadly classified into three clades and TaZnF belonged to clade I and it showed maximum similarity with OsbZIP50. Multiple alignments of the TabZIP full length protein sequence with those of the earlier reported genes showed its nearest homolog from H. vulgare. Expression analysis of TabZIP showed it to be upregulated in both roots and shoots under various abiotic stresses and under all abiotic stress such as SA, PEG, ABA, NaCl, CaCl₂ and heat stress. TabZIP functions as transcriptional activator as revealed by yeast-two-hybrid. The subcellular localization of TabZIP showed it to be localized in nucleus as well as plasma membrane. TabZIP overexpressing Arabidopsis transgenics revealed it to be responsive to salt and drought stress.

Comparative analysis

The expression profile of heat-responsive genes isolated from T. aestivum were compared with three other species, i.e. T. monococcum, A. speltoides and A. tauschii. Based on expression profiles, a distinction was possible amongst them dividing them
into four different categories. Quantitative Real-time PCR analysis revealed that expression level of these genes were significantly higher in \textit{T. aestivum} as the genes were obtained from the heat-stressed subtractive library of \textit{T. aestivum}. Southern analysis revealed certain big gene families in wheat genome (\textit{TaZnF}, \textit{TaMP}, and \textit{TaHSP26}). Further, BACs were successfully isolated obtained from \textit{T. durum} genomic library filters for five of the bread wheat genes from \textit{T. aestivum}, i.e. \textit{TaHSF}, \textit{TaMIPS}, \textit{TaMADS-box}, \textit{TabZIP} and \textit{TaZn-finger}. A 301 bp promoter of \textit{TdHSF} has been isolated using the BAC clone from \textit{T. durum}. Genomic variability was evident amongst different genome donors, both qualitatively and quantitatively.