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Identification and characterization of high temperature stress responsive genes in bread wheat (*Triticum aestivum* L.) and their regulation at various stages of development

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Abstract To elucidate the effect of high temperature, wheat plants (*Triticum aestivum* cv. CPAN 1676) were given heat shock at 37 and 42°C for 2 h, and responsive genes were identified through PCR-Select Subtraction technology. Four subtractive cDNA libraries, including three forward and one reverse subtraction, were constructed from three different developmental stages. A total of 5,500 ESTs were generated and 3,516 high quality ESTs submitted to Genbank. More than one-third of the ESTs generated fall in unknown/no hit category upon homology search through BLAST analysis. Differential expression was confirmed by cDNA macroarray and by northern/RT-PCR analysis. Expression analysis of wheat plants subjected to high temperature stress, after 1 and 4 days of recovery, showed fast recovery in seedling tissue. However, even after 4 days, recovery was negligible in the developing seed tissue after 2 h of heat stress. Ten selected genes were analyzed in further detail including one unknown protein and a new heat shock factor, by quantitative real-time PCR in an array of 35 different wheat tissues representing major developmental stages as well as different abiotic stresses. Tissue specificity was examined along with cross talk with other abiotic stresses and putative signalling molecules.

Keywords Heat stress · HSF · HSPs · Subtractive hybridization · Transcriptome · Wheat

Introduction

Climate change and abiotic stress affect agriculture and crop production adversely. Of the various climatic factors affecting agriculture, temperature is one of the most important as higher temperatures adversely affect plant growth and yield (Kurek et al. 2007). Global mean surface temperature increased by $\simeq$0.5°C in the twentieth century and is projected to rise in a range from 1.8 to 4.5°C by 2100 (Working group I report, IPCC 2007). An in depth analysis carried out by Lobell and Field (2007), involving effect of global warming on six major food crops from the year 1982–2002, revealed combined yield loss of around 40 million tonnes for wheat, corn and barley per year, where wheat alone accounts for almost half of the yield loss (19 mt year^{-1}).

At the global level, the demand for wheat is growing at approximately 2% per year (Rosegrant and Cline 2003). However, genetic gains in yield potential of wheat stand at less than 1% (Sayre et al. 1997). Consequently, an yield plateau has been observed in the last decade and has been attributed to many factors among which high occurrence of terminal heat stress appears to be the most prominent (Nagarajan 2005). A significant proportion of wheat grown in South Asia experiences heat stress out of which a major portion is present in India (Joshi et al. 2007). Wheat is grown in India under sub-tropical environment during mild

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winter which warms up towards grain filling stages of the crop. Since nearly 90% area under wheat in India has access to irrigation, yield is limited by supra-optimal temperatures prevailing during various crop growth stages. Almost all stages of wheat growth and development are adversely affected by heat stress. It has long been known that average wheat yield reduces by 4% for every one degree rise in ambient temperature during grain filling (McDonald et al. 1983). High temperature drastically reduces both yield and quality of wheat (Wardlaw et al. 2002; Altenbach et al. 2002, 2003; Dupont et al. 2006). Heat stress induce decrease in the duration of developmental phases leading to fewer organs, smaller organs, reduced light perception over the shortened life cycle and perturbation of the processes related to carbon assimilation (transpiration, photosynthesis and respiration) are significant contributing to losses of yield (Stone 2001; Rane and Chauhan 2002; Hussain and Mudasser 2007). In a recent study, Dupont et al. (2006) found that high temperatures during grain growth shortened and compressed stages of grain filling, reduced duration of dry matter accumulation and reduced kernel weight by 50%. The yield potential of a given cultivar is dependent not only on the stress sensitivity of the reproductive and grain filling stages but also on the overall plant growth and development. Efficient photosynthesis and assimilate partitioning during the vegetative phase has a decisive role in the formation of generative organs and thus directly affects the final yield. Therefore, to improve the yield potential in this changing environment, the entire development of germinating seeds to developing seeds needs exploration and redressal.

Tolerance to heat stress is a complex phenomenon and controlled by multiple genes imparting a number of physiological and biochemical changes such as alteration in membrane structures and function, tissue water content, composition of protein, lipids, primary and secondary metabolites (Shinozaki and Dennis 2003; Zhang et al. 2005; Kotak et al. 2007; Barnabas et al. 2008; Huang and Xu 2008) and no single trait fully explains why some wheat varieties are able to give better yield even when they experience heat stress. A significant increase in our understanding of molecular basis of plant stress tolerance will allow breeders to address the problem of built-in resistance against high temperature stress. Hence, there is an urgent need to elucidate molecular and genetic basis of heat tolerance in wheat, to identify beneficial genes and alleles, and to utilize them in molecular breeding programmes targeted to produce superior wheat cultivars in the future. Recent advances in genome sequencing and global gene expression analysis has led to the identification of hundreds of plant genes governing abiotic stress response (Fowler and Thomashow 2002; Kreps et al. 2002).

In wheat, although the effect of high temperature on physiology and biochemistry has been well investigated, there are only a few studies devoted to transcript profiling of wheat during high temperature stress (Altenbach and Kothari 2004; Altenbach et al. 2007, 2008; Qin et al. 2008). Moreover, almost all of these studies concentrate on the effect of high temperature during the grain growth period however, studies focusing on the effect of elevated temperature on transcriptome of seedlings and flowering stages are rare (Nagarajan 2005).

Subtractive hybridization coupled with Suppression Subtractive Hybridization (SSH) is a powerful tool for cloning differentially expressed genes and has been applied in many studies to identify abiotic stress regulated gene transcripts in plants (Boominathan et al. 2004; Liu et al. 2008). Keeping this in mind, we carried out a detailed transcriptome analysis through suppression subtractive hybridization of heat stressed and non-stressed tissues of wheat at three different growth stages, viz. young seedling, pre-pollinated flower and developing grains. Heat tolerance related 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.

Materials and methods

Plant material, growth conditions and stress treatment

Indian bread wheat (*Triticum aestivum*) cv CPAN1676, which is an intermediate type of variety for heat tolerance was used in this study and plants were grown in a growth chamber (Conviron, Canada) maintained at 20°C day:night temperature in a 16:8 h photoperiod. Plants were also grown in the departmental garden during crop season in potted soil. To reduce the environmental variation, the same experiment was conducted twice with two separate growth chambers switched for control and heat stress treatments and the plant tissues from two experiments were pooled together. The plants were arranged in a randomized complete block design within each growth chamber. For seedlings, heat stress was given by transferring 10-day-old plants to a growth chamber maintained at 37°C for 2 h and then at 42°C for another 2 h. After heat stress treatment, control and stressed seedling shoot tissues were sampled, flash frozen in liquid nitrogen and stored at −80°C until RNA isolation. Remaining seedlings were transferred back to the control growth chamber for recovery and seedling shoot tissue was again sampled after 1 and 4 days of recovery. For unfertilized flower library, heat stress was given to the potted plants after spikes were fully extended from the flag leaf sheath but prior to anthesis. Sampling of

flower tissue was done by removing lemma and palea from the spike of stressed and control plants, remaining flower organs (anther and ovary) were stored at −80°C until RNA isolation. For developing grains, potted plants grown in departmental garden were transferred for 2 h to the growth chamber maintained at 37 and 42°C at 7, 15, 21 and 30 days after anthesis followed by recovery period as above. Sampling of developing grains was done from spikes of control, stressed and recovery plants and developing seeds were stored at −80°C until RNA isolation. Similarly, for northern analysis heat stress was given at 37°C as well as at 42°C for all the tissues.

For other stress and hormone treatments, 10-day-old seedlings were used and salt stress was given by immersing seedlings in 150 mM NaCl solution, drought stress by 2% mannitol solution for 1 day. Similarly, ABA (10 μM, Sigma, USA), brassinosteroid (1 μM epibrassinolide, Sigma), salicylic Acid (100 μM) and $CaCl_2$ (10 mM) treatments were also given for 1 day. After treatment, sampling of seedling shoot tissue was done and tissue frozen and stored at −80°C.

Isolation of total RNA, mRNA and construction of subtracted cDNA libraries

Total RNA from seedling shoot and flower tissues (anthers and ovaries) were isolated by Trizole Reagent (Invitrogen) as per the manufacturer protocol. Total RNA from developing wheat seeds were isolated (stages as mentioned above) as per the method described by Singh et al. (2003). mRNA was isolated from total RNA by PolyATract mRNA isolation system (Promega, USA) as per the manufacturer's protocol. Subtracted cDNA library was constructed by using CLONTECH PCR-Select cDNA subtraction kit (CLONTECH Laboratories, USA) following manufacturer's protocol. In brief, tester (stressed) and driver (control) double stranded cDNAs were prepared from 2 μg mRNA. Tester and Driver cDNAs were separately digested with *Rsa*I to obtain shorter blunt ended molecules. Two tester populations were created by ligating two aliquots of diluted tester cDNA with two different adaptors (adaptors 1 and 2R), separately. First hybridization was performed by the following procedure. Each tester population was mixed with an excess of digested driver cDNA. The samples were heat denatured and allowed to anneal at 68°C for 8 h. The two samples from the first hybridization reaction were mixed together, and more denatured driver cDNA was added for further overnight hybridization to enrich differentially expressed sequences. Differentially expressed cDNAs, with different adaptor sequences at two ends, were selectively amplified by PCR and a second PCR was done with nested primers to further reduce the background. The subtracted and enriched DNA fragments were directly cloned into pGEMTeasy T/A cloning vector (Promega, USA). Electro competent cells of *Escherichia coli* DH5α (Invitrogen) were transformed with the ligation mix and plated on LB-agar (Pronadisa, Spain) plates containing ampicillin, isopropylthio-β galactoside, and X-gal for blue-white selection (Sambrook and Russel 2001). For seedling and unfertilized flower library, subtraction was done in forward direction, while for developing seed library, subtraction was done in both forward and reverse direction. Thus, a total of four different subtracted libraries were constructed.

Sequencing and EST analysis

For high-throughput sequencing, clones from all libraries were cultured in 96 well deepwell plates containing 1.25 ml LB broth (Pronadisa, Spain) supplemented with 100 μg ml^{-1} ampicillin for 22 h at 37°C with shaking at 200 rpm. Plasmid DNA was prepared using alkaline lysis preparation protocol (Genesis Workstation 200, TECAN, Switzerland). All sequencing reactions were conducted with the modified M13 reverse primer (5′-AGC GGA TAA CAA TTT CAC ACA GG-3′). Sequencing was performed in an ABI3700 capillary sequencer. The trace files obtained from the ABI sequencer were then entered into a pipeline that entailed a semiautomatic process to perform the base calling using Phred (http://www.phrap.org). Each sequence was screened for overall base quality and contaminating vector, mitochondrial, ribosomal, and *E. coli* sequences were removed. All sequences of the assembled EST database, singlets and contigs, were examined for homology to the NCBI nr database by BLASTX analysis (Altschul et al. 1997). For Gene Ontology (GO) term identification BLASTX was done against TIGR rice genome database to identify Locus IDs of rice homologs.

Amplification of cDNA inserts and preparation of cDNA macroarrays

Individual clones of the subtracted cDNA libraries were amplified in a 96-well PCR reaction plate using adapter primer 1 and 2R in a 50-μl reaction. PCR was conducted with the following program using Taq DNA Polymerase (Roche, Germany): initial denaturation at 94°C for 5 min, followed with 94°C for 30 s, 60°C for 30 s, 72°C for 1 min with 30 cycles. The products were analyzed in agarose gel to confirm the insert size, quality, and quantity by running with GeneRuler™ DNA size markers (Fermentas, Lithuania). PCR products were denatured by adding an equal volume of 0.6 M sodium hydroxide. Equal volume of the denatured PCR product (about 100 ng) of ≥250 bp of size was spotted on two Hybond N membranes (Amersham Pharmacia Biotech, UK) to make two identical arrays using

dot-blot apparatus (Amersham Pharmacia Biotech, UK) in 96-well format. In addition, a PCR product of wheat actin cDNA (GenBank accession no. AY663392) using primer sequences (5′-ATG GCT GAC GGT GAG GAC ATC-3′ and 5′-AGG TGC CAC ACG GAG CTC ATT G-3′) was spotted as internal control to normalize the signals of two different blots corresponding to stressed and unstressed samples. A PCR product of neomycin phosphotransferase (NPTII) gene from the vector pCAMBIA 2301 (GenBank accession no. AF234316) using primer sequences (5′-TTT TCT CCC AAT CAG GCT TG-3′ and 5′-TCA GGC TCT TTC ACT CCA TC- 3′) was also spotted as a negative control to subtract the background noise. The membranes were neutralized with neutralization buffer (0.5 M Tris–HCl, pH 7.5, 1.5 M NaCl) for 2 min, washed with 2× SSC, and cross linked using UV cross linker (Amersham Pharmacia Biotech, UK).

Probe preparation, hybridization, and data analysis

Control and stress mRNAs were labeled with α^{32}P-dATP (BRIT, India) by first-strand reverse transcription using SuperScriptIII First Strand cDNA Synthesis Kit (Life Technologies, USA). Two micrograms of mRNA was labeled in a 20-µl reaction volume containing 1× reaction buffer, 1 µL of oligo $dT_{(20)}$ primer (50 µM), 2.5 mM dCTP, dTTP, dGTP, 0.02 mM dATP, 5 µL of α^{32}P-dATP (10 mCi/ml; 3,000 Ci/mmol), and 200 units of reverse transcriptase SuperscriptIII. After incubation at 45°C for 1 h, RNA was removed by incubating with RNase H at 37°C for 20 min. Radio labeled cDNAs were purified by Sephadex G-25 column (Amersham-Pharmacia Biotech, UK) and suspended in ExpressHyb hybridization buffer (CLONTECH Laboratories, USA). Nylon membranes were prehybridized with the same buffer with 10 µg/ml of denatured herring sperm DNA for 4 h at 65°C and hybridized with denatured control and experimental cDNA probes at the same condition for 24 h. The membranes were washed three times first with 2xSSC, 0.05% SDS for 10 min and then once with 0.1× SSC, 0.1% SDS, for 10 min, at 65°C. Membranes were than exposed to BioMax MS films (Kodak, USA) with intensifying screens and stored at −80°C for 2–3 days. Images of the membranes were scanned in Typhoon (Amersham-Pharmacia Biotech, U.K.) and signal intensities were analyzed. The program allows normalization of the signal against background. Fold change was calculated as suggested by Boominathan et al. (2004).

$$\text{Fold change} = \frac{\text{Effective signal intensity in heat}}{\text{Effective signal intensity in control}} \div \frac{\text{Intensity of actin in heat}}{\text{Intensity of actin in control}}$$

Heatmap was constructed using Mayday 2.0 software (http://www.zbit.uni-tuebingen.de/pas/mayday/). The values obtained from fold change were used as input data, and four maps were constructed.

Northern hybridization

Twenty micrograms of total RNA from control and treated samples were resolved in 1.2% agarose gel containing formaldehyde and transferred to Hybond N membrane (Amersham Pharmacia Biotech, UK). PCR-amplified individual cDNA fragment (with primers corresponding to adaptor 1 and 2R) was purified from agarose gel. Probes were labeled with α^{32}P-dATP using Megaprime DNA labelling system (Amersham Pharmacia Biotech, UK) and purified through Probquant G-25 column (Amersham Pharmacia Biotech, UK). Northern hybridization was performed and band intensity calculated following the procedure described above.

Semi-quantitative RT-PCR

For expression study of wheat heat stress-related genes from unfertilized flower tissue under high temperature, a two-step RT-PCR was used in place of northern analysis because of the limited amount of the tissue available. 2 µg of mRNA from each sample was used to synthesize the first strand cDNA using the SuperScriptIII First Strand cDNA Synthesis Kit (Life Technologies, USA) in a 20 µl reaction volume as described above for cDNA labelling. After RNase H treatment, 80 µL MQ was added to each tube. 1 µl of this diluted cDNA template was added to each PCR reaction with wheat actin gene primers and gene-specific primers. Wheat gene-specific primers were designed by using GENERUNNER programme (www.generunner.com) (Supplementary Table 1). PCR was conducted with the following program using Taq DNA Polymerase (Roche, Germany): initial denaturation at 94°C for 5 min, followed with 94°C for 30 s, 60°C for 30 s, 72°C for 1 min with 30 cycles. The PCR products were checked on 1.5% agarose gel in 1× TAE buffer with EtBr. All gene-specific primers amplified cDNA fragments with corresponding sizes predicted by the designed primer. The expression level of genes in each sample were compared using actin as an internal control.

Real time expression analysis

Total RNA was isolated using the RNeasy Plant mini kit (Qiagen, Germany) according to the manufacturer's instructions, including on-column DNase I treatment to remove genomic DNA contamination. 2 µg of the total

RNA was used as template to synthesize cDNA employing the High Capacity cDNA Archive kit (Applied Biosystems, USA) and mixed with 200 nM of each primer and SYBR Green PCR Master Mix (Applied Biosystems) for real-time PCR analysis, using the ABI Prism 7000 Sequence Detection System and Software (PE Applied Biosystems) according to the manufacturer's protocol. The primer pairs were designed by using Primer Express 2.0 software (PE Applied Biosystems) and were checked by the BLAST program in wheat sequences available in the NCBI database to ensure that the primers amplify a desired unique cDNA segment (Supplementary Table 2). The specificity of the reactions was verified by melting curve analysis. The relative mRNA levels for the genes studied in RNA isolated from various tissue samples were quantified with respect to the internal control, *Actin*. At least two independent RNA isolations (biological replicates) were used for cDNA synthesis, and each cDNA sample was subjected to real-time PCR analysis in triplicate. The Ct (threshold cycles) values were averaged for three replicates and the values normalized with the Ct values of internal control *Actin*.

Results and discussion

Temperatures above optimum levels are known to impair dry matter accumulation, resulting in reduced grain weight and size in all major cereal crops, including wheat, maize, barley and rice. Studies concerning physiological and molecular effects of elevated temperatures due to global warming are thus inevitable (Yamakawa et al. 2007). We generated wheat heat stress ESTs representing three different tissues and two subtractive hybridizations and submitted a total of 3516 ESTs to public database. Data obtained were further validated by northern and dot blot analyses and some significant findings of these analyses are discussed below.

Identification and characterization of heat stress related ESTs

Four subtracted cDNA libraries, represented by a total of 5,500 clones, were constructed (Table 1); three forward subtracted libraries (FSH) contained transcripts putatively differential/up-regulated by heat stress (total 4,000 clones) and the reverse subtracted library of developing grains representing transcripts putatively down-regulated by heat stress (total 1,500 clones). The insert size in the four libraries ranged between 250 bp to 2 kb. High throughput sequencing was done for all 5,500 clones. These ESTs were assembled into 995 contigs and 666 singlets by PHRAP including CROSS-MATCH for screening vector sequences. Table 1 gives the details of contigs and singlets present in each library. These ESTs were submitted to GenBank dbEST (www.ncbi.nlm.nih.gov/dbEST), with accession number from GD186391 to GD189906. In order to annotate these contigs/singlets from clustered ESTs, protein homologies were searched in public database (NCBI and TIGR) using BLASTX and putative functions assigned. There were a number of redundant clones, indicating their abundance in the heat stressed samples, however, the largest category was of no match or proteins with unknown functions (Table 1). The group 'No hits found' constitutes around 40% of all contigs and singlets taken together.

Contigs and singlets included in all the libraries were classified separately by GO (Gene Ontology) annotations of rice. For identification of GO terms of corresponding rice homologues, all contigs and singlet reads were

Table 1 Description of different libraries

Library name	Tissue	Total EST sequenced	No. of contigs	No. of singlets	No Hit/ unknown	ESTs checked by macroarray	ESTs submitted to GENBANK database (Accession number)
Taz	10 days seedling (FSH)	1,500	201	170	145	262	846 (GD189061-GD189906)
Tau	Unfertilized flower (FSH)	1,000	209	90	165	127	546 (GD188515-GD189060)
Taw	Developing seed (RSH)	1,500	300	230	212	148	1,146 (GD187369-GD188514)
Tax	Developing seed (FSH)	1,500	285	176	149	95	978 (GD186391-GD1873680)
	Total	5,500	995	666	671	632	3,516

FSH forward subtractive hybridization, *RSH* reverse subtractive hybridization

subjected to BLAST against rice genome database (www.tigr.org) and locus ID retrieved. These locus IDs were used to assign GO terms under three main categories viz. biological process, cellular component and molecular function (http://www.tigr.org/tdb/e2k1/osa1/GO.retrieval.shtml) (Fig. 1a–c). Functional characterization through GO based Molecular Function category showed great diversity among the libraries (Fig. 1a). For seedling and flower forward subtracted libraries, more than one-third transcripts could not be assigned any known GO term, possibly due to a large number of unknown/novel genes identified in the present study. Catalytic activity makes the second largest subcategory. Transporter activity also makes a significant part for flower library and reverse subtracted developing seed library. The reverse subtracted developing seed library showed most complex picture and was subdivided into 16 subcategories, with transcription regulation, hydrolase activity and catalytic activity being the largest subcategories. However, in the forward subtracted developing seed library, transcription factor is the largest subcategory followed by no GO ID. In the biological process category, one-third of the transcripts account for stress-related subcategory in all the three forward subtracted libraries, while it was only 13% of the reverse subtracted developing seed library showing a 2-threefold increase in the number of stress-related genes by high temperature (Fig. 1b). Transcription and metabolic process makes the second and third largest subcategories, respectively, in forward subtractions. Metabolic process is the largest subcategory in the reverse subtracted developing seed library followed by transcription and carbohydrate metabolism, as expected, because developing wheat seed is a reservoir of endospermic carbohydrate (Fig. 1b). All the libraries were essentially similar for cellular component category, with nucleus, mitochondria and plastids accounting for more than 60% of total transcripts. However, the components representing cytoskeleton and endoplasmic reticulum appeared in both the developing grain

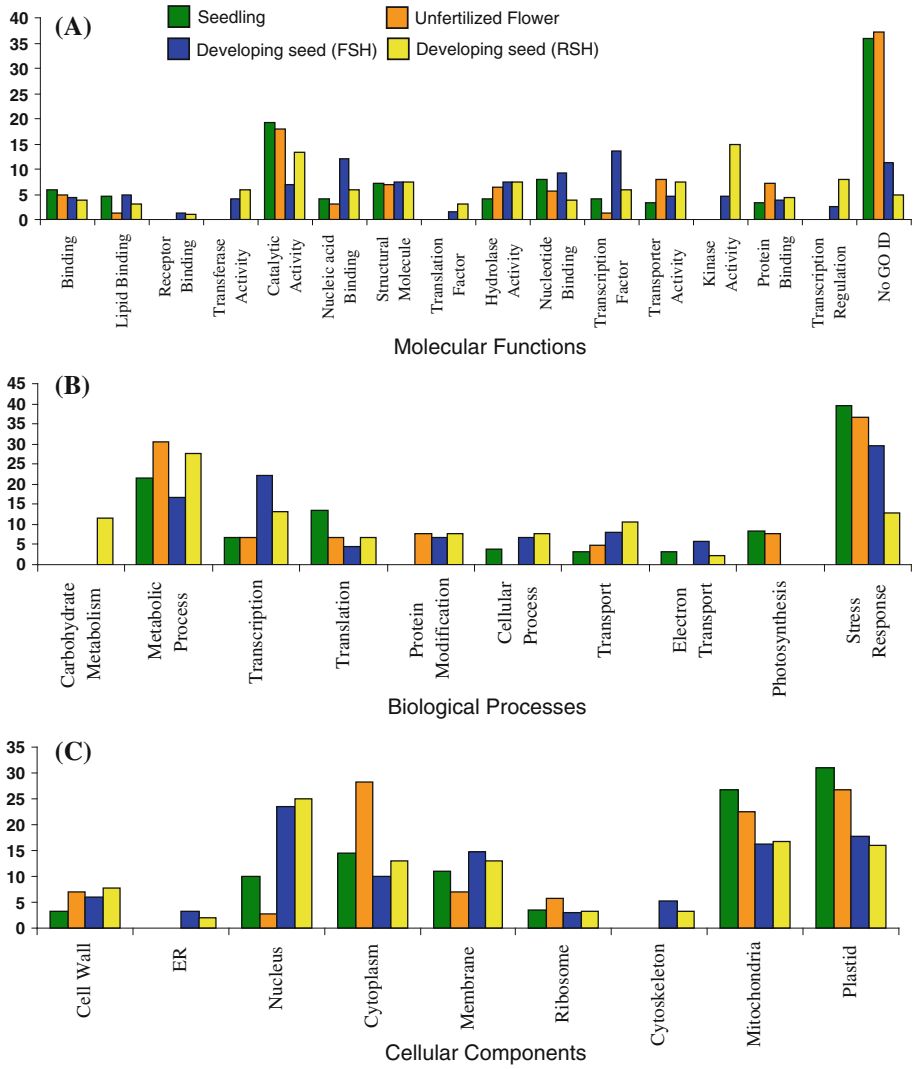

Fig. 1 Gene Ontology (GO) based classification of ESTs in different libraries

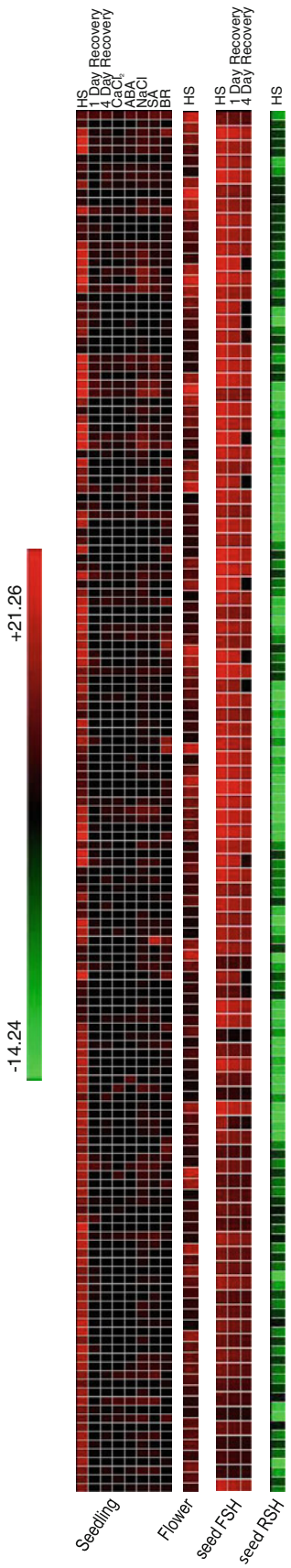

Fig. 2 Heat map representing expression analysis of ESTs in different libraries and treatments. The order of the genes is the same as given in respective Supplementary table 4–7

libraries suggesting a more elaborated role in this tissue (Fig. 1c).

To gain further insight into putative functions of ESTs from all four libraries, protein sequences of all corresponding rice unknown/hypothetical proteins based on BLASTX homology search were retrieved. These protein sequences were again searched for presence of putative conserved domain using SMART database. It was found that a majority of the proteins have one or more domains related to stress-associated functions, such as Aha1_N SNARE_assoc, FKBP_C and TPR, STI1 and UBA, SUI1, AAA, DnaJ, Inos-1-P-synth, zinc finger zf-DHHC and zf-C2HC, Usp, STI1 and TBR, along with many other conserved domains of unknown function. Supplementary Table 3 lists the library contigs, corresponding rice homolog accession numbers as well as details of the conserved domains along with their Pfam accession numbers.

A combination of approaches were employed to assess differential gene expression analysis in response to heat stress as well as to gain some insight into its regulation and signal transduction by plant hormones, and to check for cross talk with other abiotic stresses. The relative fold change was calculated by reverse northern cDNA macroarray. Figure 2 shows Heatmap generated by MAYDAY software of all the genes regulated by heat stress as well as by at least one inducer in seedling library, along with effect of heat stress in other three libraries. Of these, 178 transcripts showed more than fivefold increase after 2 h of treatment while more than 95% transcripts showed more than twofold induction (Fig. 2).The complete list of all 632 transcripts considered for reverse northern analysis is given in the Supplementary Tables 4 to 7, along with respective fold change and Genbank Accession Numbers.

Seedling response to heat stress and hormonal regulation

One-day and four-day recovery tissues along with control tissues treated with $CaCl_2$, ABA, BR, SA and NaCl were subjected to reverse northern analysis for a total of 262 transcripts of the seedling library. After 1-day recovery, only 46 transcripts showed more than twofold induction, indicating a fast recovery in the seedling tissue, while, after 4 days of recovery, 52 transcripts showed more than twofold induction, possibly due to reappearance of some genes which are important for acclimation and transcriptional reprogramming for normal growth and development (Supplementary Table 4).

Hormones are known to play an important role in plant growth and development as well as regulating the stress response. To decipher if heat stress signal transduction shares some components with hormone signalling, control tissue was treated with different hormones and reverse northern analysis was done for 262 transcripts of the seedling library. Many of the genes were commonly induced, whereas some of them displayed specific response to a particular treatment. Of the 262 genes, 42 were up-regulated by $CaCl_2$, 49 by ABA, 83 by SA and 86 by BR (Supplementary Table 4), indicating that BR and SA regulate expression of maximum number of the genes. There are many stress-related genes which are also induced by all the hormones, such as those encoding cinnamoyl CoA reductase, ferritin, peptydil prolyl isomerase (FKBP73), plasma membrane intrinsic protein (AQP2), rubisco activase B, glutaredoxin, putative LMW-HSP and many unknown genes. However, genes having specific inducer other than heat stress were also identified (Supplementary Table 4). Out of 262 transcripts checked by macroarray, 107 transcripts were also induced by salt stress (Supplementary Table 4), suggesting a cross talk between the two abiotic stresses.

Abiotic stresses cause rapid elevation in the cytosolic calcium ion concentration and this level remains considerably high even after long recovery (Knight et al. 1998; Gong et al. 1998). This change in calcium level affects the binding activity of heat stress transcription factors (HSF) to the heat shock elements (HSE) (Mosser et al. 1990). Liu et al. (2003) proposed a putative Ca^{2+} mediated signal transduction pathway for heat stress. The heat stress signals are perceived by an unidentified receptor, which upon activation increases the concentration of calcium ion through opening of calcium channels. This elevated calcium directly activates calmodulin which in turn promotes the DNA binding activity of heat shock factors, thus initiating synthesis of heat stress-related proteins such as HSPs. Very recently, Saidi et al. (2009) have found a Ca^{2+} permeable channel in the plasma membrane that is activated by high temperature and modulate the intensity of heat shock response. In the present study, we found that 16% of seedling subtractive library ESTs are also up-regulated by a 2-h treatment with 10 mM $CaCl_2$. Mainly HSPs and many calcium binding protein like TaCAM3-1, calcineurin and calnexin along with other unidentified calcium binding domain containing proteins, are induced, as revealed by the analysis of all the three forward subtractive libraries. Previous studies have shown that level of calmodulin is increased in heat stress response in maize (Gong et al. 1997) and wheat (Liu et al. 2003).

ABA is the key hormone involved in drought stress signal transduction and most of the genes that respond to drought also respond to exogenous ABA treatment (Seki et al. 2001, 2002). Since drought and heat stress frequently occur simultaneously in the field conditions in many parts of the world, ABA induction is an important regulator of thermotolerance (Maestri et al. 2002). Gong et al. (1998) found that as far as thermotolerance is concerned, ABA works synergistically with calcium. In the present investigation, we found that ABA is regulating 49 ESTs and most of them fall in the unknown category. Brassinosteroids protect plants from a variety of environmental stresses such as high and low temperatures, drought, salinity and herbicide (Khripach et al. 2000; Krishna 2003; Kagale et al. 2007). Daubhadel et al. (1999, 2002) found that exogenous application of 24-epibrassinolide increases the basic thermotolerance of *Brassica napus* and tomato seedlings. We found that among the ESTs generated from the seedling library, the largest number of ESTs (total 86) are up-regulated after a 2-h treatment with 100 nM epibrassinolide. Among the other genes, ESTs related to HSPs and detoxifiers were highly up-regulated by brassinosteroid (Supplementary Table 4). Although salicylic acid (SA) is known to take part primarily in biotic stress signalling (Heil and Bostock 2002), levels of SA were found to increase within 5 min of heat stress onset suggesting its role in heat stress signalling as well (Kaplan et al. 2004). Similarly, in the present study, SA treatment stimulated the expression of 83 out of 262 ESTs checked through cDNA macroarray. Several studies have shown that exogenous application of SA helps to confer thermotolerance in plants (Dat et al. 1998; Clarke et al. 2004). It is well documented that several steps in heat stress response pathway overlap with those involved in the response to various other forms of abiotic stresses such as drought and cold (Zhu 2001; Rizhsky et al. 2004; Yamaguchi-Shinozaki and Shinozaki 2006; Swindell et al. 2007). The present data suggest that salinity is also not an exception in this respect. Thus, it is evident that the heat stress signal transduction is quite complex. There is a possibility of many parallel heat stress transduction pathways being governed by specific hormones or there could be a single pathway and different hormones play specific roles in the network.

We also checked 36 gene transcripts from the seedling library by northern analysis for their response at 37 and 42°C (Fig. 3). These genes were selected from different functional categories, such as molecular chaperons and HSPs, transporters, protein modifiers, signalling molecules, stress-related and unknown functions. Figure 3 shows that most of these genes are highly inducible by high temperature and remain stable at both temperature regimes. However, there are a few which have greater expression at 42°C. Two of such genes encode unknown proteins and may be of special interest as they are induced at 42°C only, suggesting their specific role during severe heat stress. Another such gene is Rubisco Activase B (RcaB), which is induced at 37°C, but its transcript is relatively reduced at 42°C.

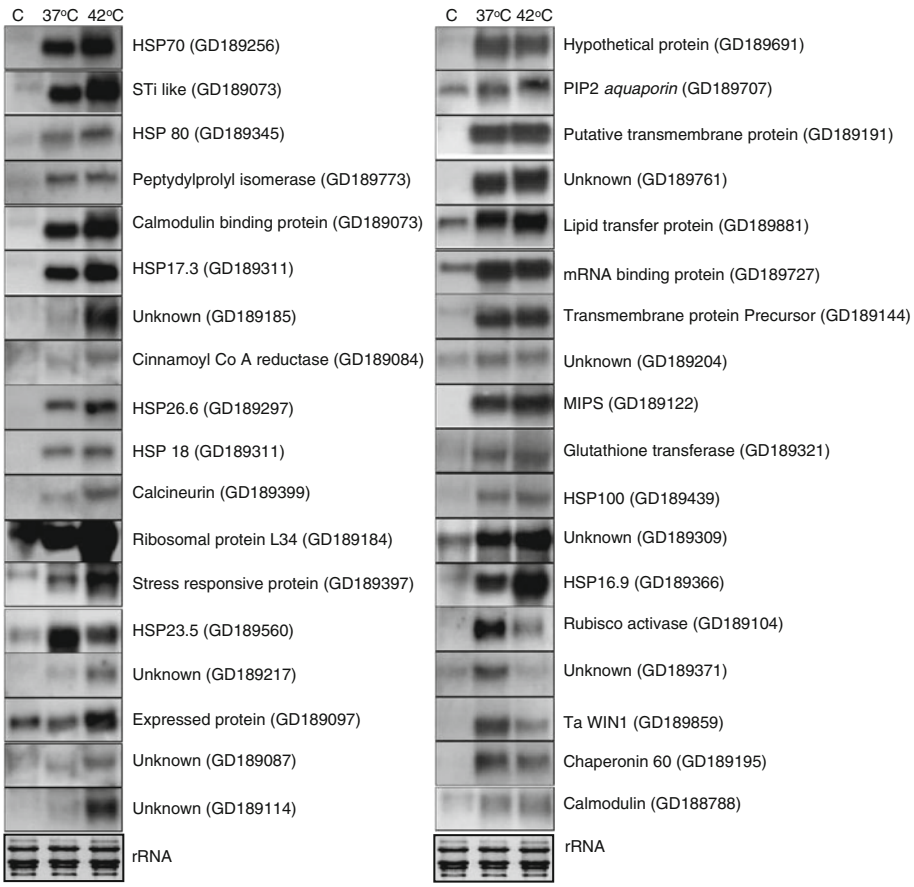

Fig. 3 Northern analysis of some ESTs from Taz (Seedling FSH) library. Seedlings were given heat stress at 37 and 42°C

Transcripts of many genes, such as HSPs, lipid transfer protein, L-*myo*-inositol-1-phosphate synthase (MIPS), cpn60, calcium binding proteins and membrane binding proteins were observed to be induced and stable at 37 and 42°C. Calcineurin is a Ca^{2+} and calmodulin dependent serine/threonine phosphatase and its expression is observed during various abiotic stresses in *Arabidopsis* (Kudla et al. 1999). Nonspecific lipid transfer proteins have also been seen to increase by both biotic and abiotic stresses as they protect plants by formation of a hydrophobic layer (Trevino and Ma 1998). L-*myo*-inositol-1-phosphate synthase (MIPS) is known to protect plants from salt and drought stress (Boominathan et al. 2004; Majee et al. 2004), and under transgenic conditions provide higher photosynthetic competence and better growth during salinity stress.

Expression profiles of ten selected genes from four libraries in 35 different wheat tissues sampled on the basis of various growth stages and stress treatments were also monitored by real time PCR (Fig. 4). Among these genes, one unknown putative membrane protein encoding gene (chosen from seedling library) was found to be seedling specific and besides heat stress, was also regulated by other stresses, brassinosteroids and ABA. This contig (Taz Con 95) harbours a full-length EST having an ORF coding for 71 amino acids. The deduced amino acid sequence indicated the protein to be highly hydrophobic with one uncharacterized transmembrane domain UPF0057. Substantial transcript amount was present even after 4 days of heat stress recovery, suggesting its role in stress amelioration and recovery (Fig. 4a). We chose two heat shock proteins for detailed analysis by real time PCR, i.e. HSP 80 and a chloroplastic small HSP 26. Both these HSPs were present in seedling, flower and in developing seed libraries and induced by heat stress as well as by other stresses in different tissues but not present after 4 days of recovery in root and shoot tissues (Fig. 4b, c). Interestingly, HSP 26 transcript was found in very high amount in the heat-stressed flower and developing seed tissues.

A number of ESTs generated in the present study represent the proteins related to protein synthesis, such as initiation and elongation factors, protein disulphide isomerases, ADP-ribosylation factor, ribosomal proteins, Clp protease and proteosome subunits, nucleic acid binding proteins, signal transduction molecules and transcription factors, and proteins involved in various metabolic pathways like formate dehydrogenase, beta amylase and triose phosphate isomerase. Heat stress is known to result in misfolding of newly synthesized proteins and the denaturation of existing proteins. Chaperon functions, the prevention of denaturation by misfolding or the refolding of already denatured proteins

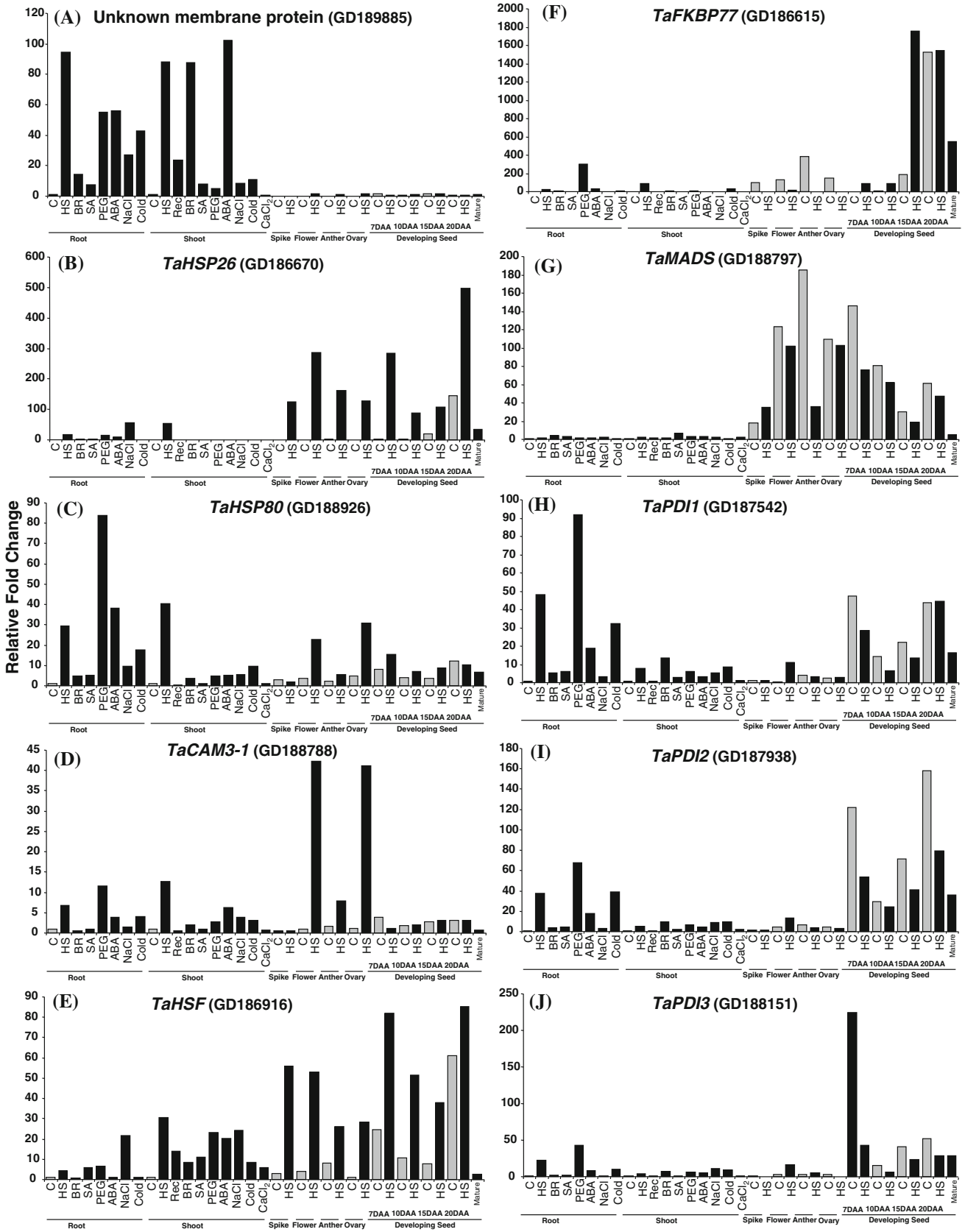

Fig. 4 Expression analysis of 10 selected genes in 35 different wheat tissues by quantitative RT-PCR

is one of the major functions of HSPs (Klueva et al. 2001). In our study, HSPs make the single largest contribution in seedling heat stress subtractive library, accounting for nearly 20% of total ESTs and we found that almost all the HSPs are highly represented in developing seed forward subtractive library also. Although in vivo function of HSPs is largely unknown, in vitro experimentation suggests that cytosolic small HSPs function as molecular chaperones by preventing thermal aggregation of proteins and facilitate their reactivation after stress (Lee et al. 1995, 1997). Majoul et al. (2004) while working on proteomics of wheat seed development under heat stress found the constitutive accumulation of small HSPs, but a clear increase in their relative protein abundance upon stress treatment. Small HSPs are known to protect developing embryo from heat stress as it is not responsive to heat stress on its own (DeRocher and Vierling 1994).

Rubisco is the enzyme that converts CO_2 into plant biomass. Its activation in light is regulated by stromal enzyme Rubisco Activase (Salvucci and Ogren 1996). High temperature is well known to inhibit photosynthetic CO_2 fixation in many plants, due to temperature induced decrease in the activation state of Rubisco (Law and Crafts-Brandner 1999; Salvucci and Crafts-Brandner 2004a, b). We found that in wheat, at least one isoform, RCAb, is heat induced (Fig. 3) as revealed by dot blot as well as by northern analysis. Recently, an association has been reported between heat-induced chloroplastal cpn60 and rubisco activase (Salvucci 2008) and a positive significant correlation was found between the expression of a wheat RCA and yield under heat stress environment (Ristic et al. 2009). Kurek et al. (2007) produced thermotolerant transgenic *Arabidopsis* plants by over-expressing a heat stress tolerant RCA, thereby validating the role of RCA in acquired thermotolerance.

Flower response to heat stress

In the flower forward-subtracted (FSH) library, all the 127 transcripts showed rapid induction after 2 h of heat stress (Supplementary Table. 5). Highest induction was shown by an unknown protein gene (21-fold induction) followed by a wheat calmodulin (TaCAM3-1), C3HC4 type zinc finger protein, 14-3-3 protein, chitinase and wheat HSP82 (wheat HSP90 homolog) (Supplementary Table 5). Additionally, these 127 genes were also tested for anther or ovary specificity through the macroarray probed with anther or ovary heat stressed RNA. We found that majority of these 127 genes have common induction in anther and ovary, but there are some genes which have differential/altered induction pattern (Supplementary Table 5). Figure 5 shows genes selected from unfertilized flower subtracted library. We have checked a total of 19 genes through RT-PCR analysis for their response towards heat stress and their specificity, if any for anther and ovary tissues. Most of these genes are present in both the tissues, however, a putative translation factor is specific to ovary and a lipid transfer protein (LTP-2) is more abundant in anther tissue. Recently, a lipid transfer protein of *Arabidopsis thaliana* (AtLTP5) has been functionally characterized and pollen-targeted overexpression of Wild-Type LTP5 or ltp5-1resulted in adverse effects on pollen tube tip growth and seed set (Chae et al. 2009). Transcription factors, such as b-ZIP and zinc finger proteins, along with a calmodulin (TaCAM3-1) are highly expressed upon high temperature stress (Supplementary Table 5). We have also checked the relative transcript abundance of this wheat calmodulin by real time PCR analysis and found that apart from different flower tissues, a significant amount of its transcript was also present in drought-stressed root and shoot tissue but was totally absent from various developing seed tissues (Fig. 4d). Another heat inducible gene is a 14-3-3 protein, which is more inducible in ovary tissue than the anthers (Fig. 5). There are several reports to show that 14-3-3 proteins are involved in plants response to environmental stress such as stress induced senescence and increased drought tolerance (Yan et al. 2004) and salt and cold stress (Chen et al. 2006). Another such gene is a C3HC4 RING finger protein, which is heat inducible in anther tissues and

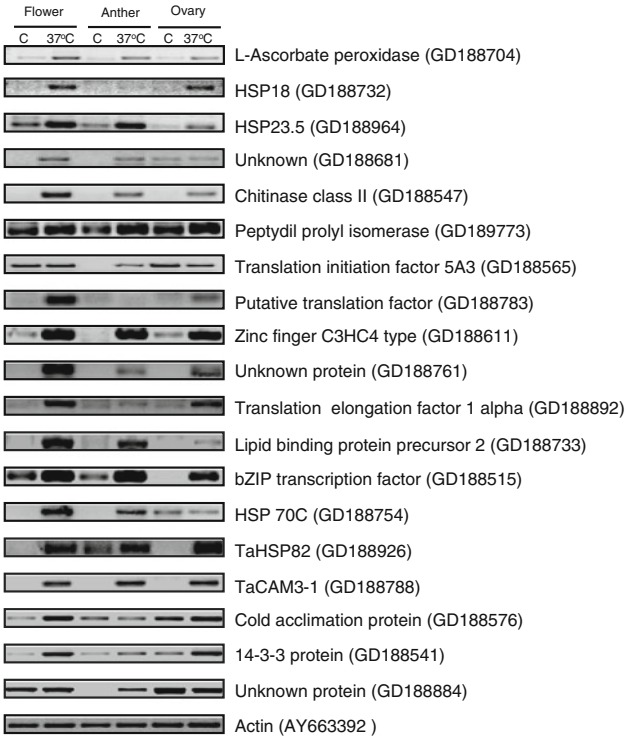

Fig. 5 Semi-quantitative RT-PCR analysis of some ESTs from Tau (Unfertilized Flower FSH) library. Heat stress was given at 37°C and actin is taken as internal control

relatively constitutive in ovary tissue (Fig. 5). These proteins have been studied in both *Arabidopsis* and rice (Stone et al. 2005; Ma et al. 2009) and found to be responsive to abiotic stresses. Another transcription factor identified from flower library is a bZIP protein, which is inducible by heat stress (Fig. 5). In plants, bZIP transcription factors control many processes such as ABA signalling, seed and flower development (Jakoby et al. 2002) and are involved in imparting stress tolerance (Fujita et al. 2005; Zhang et al. 2008).

Floral abnormalities induced by heat stress leading to spikelet sterility are known in rice (Takeoka et al. 1991). Saini and Aspinall (1982) reported that temperature above 30°C during floret formation causes complete sterility in wheat. Sinclair and Jamieson (2006) also highlighted that grain number in wheat is highly dependent on environmental conditions prior to and during flower formation. Comparative studies have shown that high temperatures cause more damage to anthers as compared to ovaries, and pollen formation is one of the most heat sensitive developmental stage in cereals in general and in wheat in particular (Saini and Aspinall 1982; Saini et al. 1984). In barley, Abiko et al. (2005) have shown through serial analysis of gene expression that high temperature results in the transcriptional inhibition of genes that are active in anther under normal temperature conditions. We have observed that 148 ESTs are affected by heat stress treatment in unfertilized wheat flower and confirmed their expression by dot blot and RT-PCR analysis. Apart from many unknown proteins, we also found a large number of transcription factors up-regulated by high temperature, which are being further characterized in our laboratory.

Developing seed and heat stress

Forward Subtraction

The macroarray representing developing seed forward subtracted library with 95 transcripts, showed highest induction by high temperature stress among all the libraries made in this study. These transcripts correspond to many stress-related proteins, such as helicase-like protein, alanine amino transferase, myo-inositol 1-P-synthase (MIPS), stress-induced protein Sti-1, activator of HSP90, peptydyl prolyl isomerase, heat shock factor (HSF), along with all types of HSPs. Supplementary Table 6 provides details of fold induction of these genes during heat stress and after recovery of 1-day and 4-day. It is clear from Supplementary Table 6 and Fig. 2 that, in the case of developing seeds, recovery is not as fast as in the seedling, and almost all the 95 genes show more than twofold induction even after 4 days of recovery period. Contrary to this, only 20% of seedling ESTs showed more than twofold increase in the transcript levels after 1-day of recovery.

For developing seed forward subtractive library, 50 genes were checked by northern analysis (Fig. 6). As expected, most of the HSPs have a basal constitutive expression in developing seed tissue, but transcript levels of almost all are enhanced at 37 and 42°C treatment given for 2 h. Along with many unknown proteins, a novel seed specific heat shock factor (HSF) and a putative helicase inducible by heat stress could be identified in developing seed tissue. This heat shock factor from developing seed forward subtractive library was also checked by real time analysis and found to be induced by calcium and heat stress, particularly in various flower and seed tissues, and significant amounts were also present in 4 day recovery tissue and 20 DAA control seed, suggesting its dual role in stress amelioration and as well as in seed maturation (Fig. 4e). This seed-specific HSF is being further characterized. Peptidyl prolyl *cis-trans* isomerases (FKBP) are known to protect plants from abiotic stresses and a heat inducible FKBP77 is known to express in wheat roots (Dwivedi et al. 2003; Geisler and Bailly 2007). We thus monitored the expression of wheat FKBP77 in different wheat tissues and found its expression induced in shoot and root tissue by heat as well as by drought stress. However, FKBP77 transcript is severely reduced in flower tissue upon heat stress but, its expression was induced again during early stages of seed development (7 and 10 DAA). At 20 DAA stage, the expression was almost same for control and heat stressed developing seeds and a significant amount was also present in the mature seed (Fig. 4f).

While studying the effect of high temperature in rice grain development, Yamakawa et al. (2007) reported on an average 89% reduction in the activities of starch biosynthesis enzymes and translocaters, while there is considerable increase in the transcripts of HSPs and starch consuming α-amylase coding gene. High temperature not only advance but also compress the normal seed development in wheat. Studies have shown that high temperature enhances apoptosis in developing wheat grains and hastens the activity of various seed specific genes (Hurkman et al. 2003) and early deposition of various gluten proteins, reducing the overall weight and size of wheat grains (Altenbach et al. 2003). Employing a proteomic approach, Hurkman et al. (2009) analyzed the effect of heat stress in developing wheat grains. They also found early and higher accumulation of proteins related to stress and plant defense pathways while proteins related to biosynthesis and metabolism decreased under heat stress. In the present study we also find that even 2 h heat treatment during grain development caused irreparable damage and accumulation of stress associated transcripts during the recovery phase. Additionally, we report involvement of a large number of

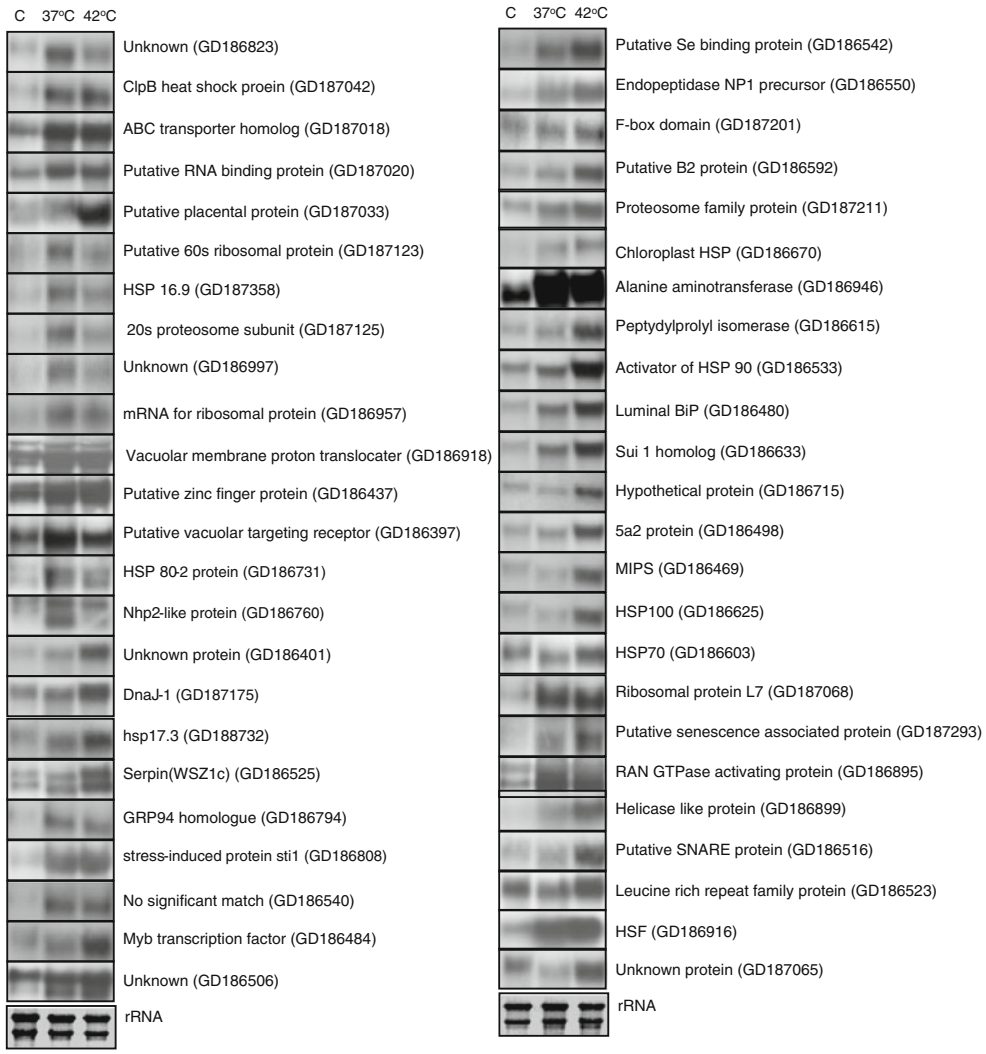

Fig. 6 Northern analysis of some ESTs from Tax (Seed FSH) library. Heat stress was given at 37 and 42°C for 2 h

novel uncharacterized transcripts in wheat heat stress response in three different tissues. One such gene is a putative HSF, which is highly induced in developing seed by heat stress. The *Arabidopsis* genome has 21 members of HSFs belonging to 3 classes and 14 groups (Nover et al. 2001). Out of 24,000 genes, 11% genes of *Arabidopsis* showed regulation during heat stress and many of these genes proved to be controlled by HSF1a/1b, as determined by global gene expression analysis (Busch et al. 2005). However, whether the HSF reported in this study has similar functions to play in wheat is yet to be determined.

Reverse subtraction

A total of 148 transcripts of developing seed reverse subtracted library were checked for down-regulation by heat stress. As expected, severely affected genes are those involved in carbohydrate metabolism, encoding components like sucrose synthase, amylase inhibitor, triose phosphate isomerase and soluble starch synthase. We found a large number of genes encoding seed storage proteins (gliadins and glutanins) affected by high temperature (Supplementary Table 7). As seen in other libraries too, there are a large number of unknown genes which may be involved in seed development and down-regulated by heat stress.

We also checked many seed storage as well as starch synthesis associated genes from reverse subtracted developing wheat grain library by northern analysis (Fig. 7). Most of the storage protein genes selected for northern analysis showed decrease in transcript abundance at 37 and 42°C (for 2 h), however, the expression of a few genes encoding glutenin subunits is relatively stable during heat stress. Protein disulfide isomerases are necessary cellular proteins for sorting and trafficking and three wheat protein disulfide isomerase encoding genes are negatively affected by high temperature (Supplementary Table 7). For all the

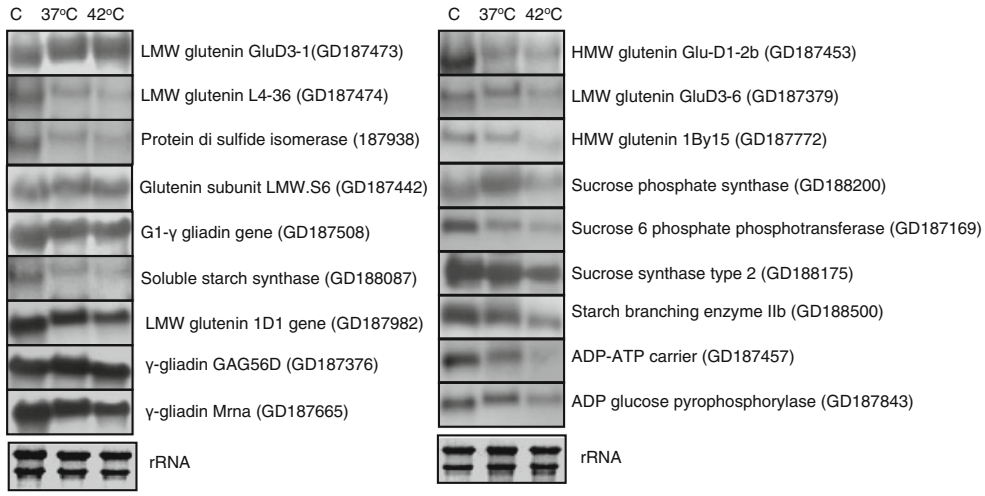

Fig. 7 Northern analysis of some ESTs from Taw (Seed RSH) library. Heat stress was given at 37 and 42°C for 2 h

starch synthesis enzymes checked in the present study, heat stress response showed a decrease in transcript abundance at 37 and 42°C, while the transcripts of starch branching enzyme IIb and sucrose synthase type II were comparatively stable at 37°C. One MADS box protein identified in this study from developing seed reverse subtraction was found to be down-regulated by heat stress in flower and developing seed tissues. Its expression was particularly reduced in the anther tissue upon heat stress, however, in the ovary it was rather stable under heat stress (Fig. 4g). Foldases are important for the final dough making quality of wheat flour (Johnson et al. 2001). We have studied expression of three genes encoding protein disulphide isomerases (PDI) along with one peptidyl prolyl *cis-trans* isomerase (FKBP77). All the three PDI encoding genes studied behaved almost similarly in all the tissues and their expression was significantly reduced in developing seed upon heat stress (Fig. 4h–j). Interestingly, all these three PDI genes were highly up-regulated by drought stress in root tissue.

In rice, Yamakawa et al. (2007) reported 68% decrease in the transcript levels of protein disulfide isomerase, an enzyme necessary for precise sorting of storage proteins. We have identified three wheat protein disulfide isomerase negatively affected by high temperature (Fig. 4h–j). Additionally, the transcript levels corresponding to all the major enzymes responsible for starch biosynthesis were found to be decreased under heat stress, as also reported earlier in wheat (Hurkman et al. 2003).

Conclusions

The demand of wheat is increasing at a rapid pace and by the year 2017 additional 200 million tonnes/year of wheat and corn amounting to additional 6 million ha of corn and 4 million ha of wheat, would be required to meet the demand (Edgerton 2009). Despite the importance of wheat, current information of its genome sequence is not sufficient for functional genomics analysis (Gill et al. 2004). The complete sequencing of the wheat genome is challenging because of its large genome size (16,000 Mb; Bennett and Leitch 2005). Nevertheless, 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 stress response (Ramalingam et al. 2006; Barnabas et al. 2008). Through SSH libraries we have generated a collection of heat stress responsive genes critical for various growth stages in wheat. This study will act as a foundation for further work in the field of wheat genomics for overcoming high temperature stress. Currently, the ESTs of many of the heat responsive genes identified in this study are being completed using 5′ and 3′ RACE, with the aim to functionally validate them through transgenic technology.

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