Characterization and Expression of High Temperature Stress Responsive Genes in Bread Wheat (*Triticum aestivum* L.)

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Abstract: To elucidate the effects of high temperatures, wheat plants (*Triticum aestivum* cv. CPAN 1676) were given heat shocks at 37°C and 42°C for two hours, 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 5500 ESTs were generated and 3516 high quality ESTs were submitted to Genbank. More than one third of the ESTs generated fall in unknown/no hit categories upon a homology search through BLAST analysis. A large number of high temperature responsive genes have been identified and characterized. Reverse subtraction analysis in developing grains showed extensive transcriptional changes upon heat stress as revealed by comparative analysis with forward subtraction. Differential expression was confirmed by cDNA macroarray and by northern/RT-PCR analysis. Expression analysis of wheat plants subjected to high temperature stress, after one and four days of recovery, showed fast recovery in seedling tissues. However, recovery was small in the developing seed tissue after two hours of heat stress. Ten selected genes were analysed in further detail 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. The results obtained contribute towards understanding the regulation of genes at different developmental stages in wheat crucial to withstanding and recovery from heat stress.

Keywords: abiotic stress; heat stress; transcriptome analysis; wheat

The demand for wheat is increasing at a rapid pace, and by the year 2017 an additional 200 mil t/year of wheat and corn, amounting to an additional 6 mil ha of corn and 4 mil ha of wheat, would be required to meet the demand. Abiotic stresses are the major limitations in the changing climatic scenario. Despite the importance of wheat, current information on its genome sequence is not yet sufficient for detailed functional genomics analysis. The complete sequencing of the wheat genome is challenging because of its large genome size (17 000 Mb). 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 responses.

Tolerance to heat stress is a complex phenomenon and controlled by multiple genes imparting a number of physiological and biochemical changes such as alterations in membrane structures and function, tissue water content, composition of protein, lipids, primary and secondary metabolites (Shinozaki & Dennis 2003; Zhang *et al.* 2005; Dupont *et al.* 2006; Barnabas *et al.* 2007), and no single trait fully explains why some wheat varieties are able to give higher yields even when they experience heat stress. In wheat, although the effect of high temperature

on the physiology and biochemistry has been well investigated, there are only a few studies devoted to transcript profiling of wheat during high temperature stress (Altenbach & Kothari 2004; Altenbach *et al.* 2007, 2008). High temperatures reduce the duration of the developmental phases leading to fewer organs, smaller organs, and affect processes related to carbon assimilation. Heat stress induces both a decrease in yield and quality of wheat. It has been reported that high temperatures during grain filling shortens and compresses the stages of grain filling, reduces the duration of dry matter accumulation, and reduces kernel weight. However, most of these studies concentrate on the effects of high temperatures during the grain growth period, and the effect of elevated temperatures on the transcriptome of seedlings and flowering stages is lacking. Therefore, 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, was undertaken. Heat tolerance related gene transcripts were identified based on their putative functions and validated by cDNA macroarray and northern/RT-PCR analysis helping to unravel the complexities of heat stress response in wheat.

Identification and characterization of heat stress related ESTs

Total RNA from seedling and flower tissue was isolated by Trizole Reagent (Invitrogen) as per the manufacturer's protocol. mRNA was isolated from total RNA by the PolyATract mRNA isolation system (Promega, USA) as per the manufacturer's protocol. A subtracted cDNA library was constructed by using a CLONTECH PCR-Select cDNA subtraction kit (CLONTECH Laboratories, USA) following manufacturer's protocol. The subtracted and enriched DNA fragments were directly cloned into pGEMTeasy T/A cloning vector (Promega, USA). For seedling and unfertilized flower libraries, subtraction was done in a forward direction, while for a developing seed library, subtraction was done in both forward and reverse directions. Thus, a total of four different subtracted libraries were constructed. Sequencing was performed in an ABI3700 capillary sequencer. 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).

Individual clones of the subtracted cDNA library were amplified in a 96-well PCR reaction plate using adapter primers 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 by 94°C for 30 s, 60°C for 30 s, 72°C for 1 min with 30 cycles. The products were analyzed in agarose gels to confirm the insert size, quality, and quantity. PCR products were denatured by adding an equal volume of 0.6M sodium hydroxide. Equal volumes of the denatured PCR product (about 100 ng) of ≥ 250 bp of size were 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 (Figure 1). In addition, a PCR product of the wheat actin cDNA using primer sequences was spotted as an internal control to normalize the signals of two different blots corresponding to stressed and non-stressed samples. A PCR product of the neomycin phosphotransferase (NPTII) gene from the vector pCAM-

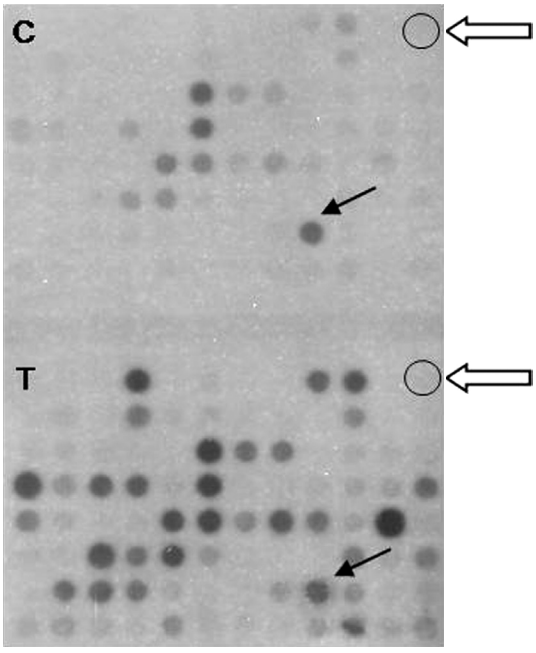

Figure 1. A representative cDNA Marco vArray probed with labelled first strand cDNA from control (C) and heat treated (T); ⇦ NPTII; ← Actin

BIA2301 was also spotted as a negative control to subtract the background signal. The membranes were neutralized with neutralization buffer (0.5M Tris-HCl, pH 7.5, 1.5M NaCl) for 2 min, washed with 2 × SSC, and cross linked using UV cross linker (Amersham Pharmacia Biotech, UK). Radio labeled cDNAs were purified by Sephadex G-25 column (Amersham-Pharmacia Biotech, UK) and suspended in ExpressHyb hybridization buffer (CLONTECH Laboratories, USA). Membranes were then exposed to BioMax MS films (Kodak, USA) with intensifying screens and stored at –80°C for two to three days. Approximately 185 ESTs were seen to be influenced by heat stress treatments in the unfertilized wheat flower library and their expression was later confirmed by dot-blot (Figure 1) and RT-PCR analysis.

Contigs and singlets were classified by Gene Ontology (GO) annotations of rice. Functional characterization through the GO based Molecular Function category showed great diversity among the libraries. For seedling and flower forward subtracted libraries, more than one third of transcripts could not be assigned to any known GO term. Catalytic activity transcripts made up the second largest subcategory. Transport activity transcripts also made up a significant part of the flower library and reverse subtracted developing seed library. The reverse subtracted developing seed library showed the most complex picture and was subdivided into 16 subcategories. However, in the forward subtracted developing seed library, transcription factors were the largest subcategory followed by no GO ID. In the biological process category, one third of the transcripts account fit into the stress-related subcategory in all the three forward subtracted libraries. Metabolic process transcripts made the second largest subcategory accounting for around one-fourth of the transcripts in forward subtractions. The components representing cytoskeleton and endoplasmic reticulum appeared in both the developing grain libraries suggesting a more elaborated role in this tissue.

Differential expression of selected genes

The role of 36 gene transcripts was also checked from the seedling library by northern analysis for their response to higher temperatures. These genes were selected from different functional categories, such as molecular chaperons and HSPs, transporters, protein modifiers, signaling molecules, stress-related and unknown functions. Most of these genes were highly inducible by high temperature and remained stable at both temperature regimes. However, there were a few which had greater expression at 42°C. The expression profile of 19 genes was also undertaken through RT-PCR analysis for their response towards heat stress and their specificity. Transcription factors, such as b-ZIP and zinc finger proteins along with a calmodulin (TaCAM3-1), were highly expressed upon high temperature stress (Chauhan *et al.* 2011).

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. We observed that 148 ESTs were affected by heat stress treatment in unfertilized wheat flowers 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.

CONCLUSIONS

Through SSH libraries we have made a collection of heat stress responsive genes critical for wheat growth stages. Four subtractive cDNA libraries, including three forward and one reverse subtraction, were constructed from three different developmental stages. Selected genes were analyzed 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. 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 ultimate aim to functionally validate them in transgenics.

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