Terminal heat stress-responsive genes analysis in heat susceptible HDR77 genotype of wheat (*Triticum aestivum* L.) by using semi-quantitative RTPCR

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Abstract

Abiotic stresses particularly heat stress constitutes a serious threat to the world food production in bread wheat. In the present study, candidate gene expression analysis of HDR77 genotype (which is a susceptible genotype based on electrolyte leakage experiment) was carried out and only those candidate genes which were showing two fold change differences were selected from previous research papers These genes were run over cDNA of HDR 77 genotype used as template and intensity of bands was checked by semi-quantitative RT PCR. Few genes like HSP 80 gene, MADSWP12 gene, HSP 26.6B gene, WRKY53b gene, WRKY72-a gene, HSP26 gene and WRKY72b gene were upregulated whereas TaVRT-MADS gene, NAC gene and WRKY10 gene were down-regulated. The heat stress responsive genes will increase our understanding of molecular basis for heat tolerance in different wheat genotypes and future improvement of heat tolerance in wheat and other crops.

Key words: Heat stress, semi-quantitative RT PCR, up-regulated, down-regulated.

Introduction

Wheat is an essential winter cereal crop of India. The net total wheat production for 2014-15 was approximately 86.53 million tonnes. This production of wheat was lower than 2013-14 which was 95.85 million tonnes (Indian wheat database). Improper agronomic practices, poor management and unfavorable weather conditions such as high temperature, drought and salinity leads to a lesser wheat production (Anonymous, 2010). Most of the crops are exposed to heat stress during certain periods of their life cycle (Stone, 2001). An exposure to higher temperature than optimal temperature decreases yield and reduces quality of cereals. As the world population is exponentially growing, there is a greater demand to increase the productivity of the crop. These goals need significant plant breeding methods for the improvement of cereal yield and quality under high temperatures. Thus, there is a great need to identify molecular and genetic basis of heat tolerance in cereals to identify beneficial genes and to use them in the molecular breeding programme to produce superior cereal cultivars in near future.

Heat stress is often defined as the rise in temperature beyond a threshold level for a period of time sufficient to cause irreversible damage to plant growth and development. In general, a transient elevation in temperature, usually 10–15°C above ambient, is considered as heat shock or heat stress. Heat stress during reproductive and grain-filling phases is termed as terminal heat stress (Khajuria et al., 2016). Heat stress affects particularly during reproductive and grain-filling phases. Heat stress is a major yield limiting factor adversely affecting wheat yield in many regions of the world (Modarresi et al. 2010). The high temperatures during the post-heading stages affect yield (Wardlaw and Willenbrink, 1994) and grain quality (Blumenthal et al., 1995) of wheat. (Heat stress leads to few number of organs, smaller organs, reduced light perception over the shortened life cycle, and perturbation of the processes related to carbon assimilation like transpiration, photosynthesis and respiration are significant for losses in cereal yields (Stone 2001).)

One of the consequences of high temperature in plants is oxidative damage caused by the heat-induced imbalance of photosynthesis and respiration. Elevated temperatures may reduce the activities of antioxidant enzymes, as observed in maize. Heat stress affects all aspects of plant processes like germination, growth, development, reproduction and yield (Lobell and Field, 2007). The optimum temperature for wheat anthesis and grain filling ranges from 12 to 22 °C. Exposure to temperatures above this can significantly reduce grain yield (Mullarkey and Jones 2000). Heat stress during anthesis increases floret abortion (Wardlaw et al. 2002). Heat stress during the reproductive phase can cause pollen sterility, lower CO₂ assimilation and increased photorespiration.
Heat stress also affects calcium signalling, sugar signalling, RNA metabolism in plants by producing a number of HSPs which are either down regulated or upregulated during heat stress (Dandan et al., 2008).

At the molecular level, heat stress causes changes in gene expression involved in direct protection from heat stress (Shinozaki and -Shinozaki, 2007). These involve genes responsible for the expression of osmoprotectants, detoxifying enzymes and regulatory proteins (Semenov and Halford 2009). Gene expression changes gradually and leads to the development of heat tolerance in the form of acclimation or to adaptation (Moreno and Orellana, 2011). The induction of heat shock proteins when plants are exposed to increased temperature has been well reported (Kotak et al., 2007; Lee et al. 1995; Ogawa et al. 2007). HSPs acts as molecular chaperones in maintaining homeostasis of protein folding and are related to the acquisition of thermotolerance (Rodriguez et al. 2005). Recently, molecular approaches have included omics techniques. This opens the way for developing stress tolerant varieties and to grow agriculturally important crop plants under heat stress. The purpose of this study was the identification of candidate genes for heat stress and candidate genes- based gene expression analysis for heat stress in bread wheat.

Materials and Methods
The research work was undertaken at the experimental farm, SKUAST-Jammu and molecular biology laboratory of School of Biotechnology, SKUAST-J, Main Campus, Jammu. The experiments were conducted during 2013-16. The experimental material for the study comprised of HDR77 genotype of wheat (Triticum aestivum L.) grown in the experimental field of SKUAST-Jammu. The genotype was sown under two environmental conditions of sowing i.e. normal sowing and late sowing with a difference of one month at SKUAST J, Chatha. Recommended package of practices were followed for raising a good crop

Previous studies having reported genome-wide gene expression results for abiotic stresses including heat were screened for the identification of potential candidate-genes showing differences in expression patterns. Priority was set on those genes that showed at least two-fold expression level differences. Full length conserved domain sequences (CDS) for the respective candidate genes from Triticum aestivum were downloaded from NCBI data base entries and primers were designed for PCR amplification using Primer 3 software (bioinfo.ut.ee/primer 3-0.4.0). Ten primer pairs were used for the amplification of corresponding cDNA fragments from a wheat genotype (HDR 77) grown under normal (timely sowing) and stressed conditions (late sowing). The information about primer pairs for complete CDS of gene sequences is given in Table 1. Another primer pair for a housekeeping gene (actin) was also included to identify the expression patterns of candidate genes.

The tissue samples of a variety (HDR 77, a susceptible genotype based on electrolyte leakage experiment) sown under normal- and heatstressed conditions were collected to identify differences in the expression of candidate genes due to heat stress.

The flag leaf of wheat variety (HDR 77) was collected when twenty percent spikes came out of flag leaf from normal condition (early sowing) as well as from stress condition (late sowing) and stored in liquid nitrogen before undertaking total RNA isolation using trizol reagent (Invitrogen). The total RNA was converted to complementary DNA (cDNA) to be used for PCR through 1st Strand cDNA synthesis kit for RT-PCR (AMV). The quality and size of first strand cDNA products was determined with gel electrophoresis on 1.5% agarose gel with actin primer (housekeeping gene) (Fig. 1). Reverse transcription polymerase chain reaction (RT PCR) was used to study the effect of terminal heat stress on the expression of the genes through semi-quantitative RT-PCR.

PCR reactions were set up in 0.2 ml thin-walled PCR tubes. PCR tubes containing master mix and the cDNA template were thoroughly mixed and subjected to thermal profile. The reaction was carried out in a gradient thermocycler (peqlab/ www.sigma-svi.com) PCR. The programme used in the thermocycler for cDNA amplification is as follows; initial denaturation for 3 min at 94°C, followed by 35 cycles of 30 s at 94°C (for denaturation) and 56°C to 59.5°C (accordig to primer annealing temperature), 72°C (for extension) at 30 s with a final extension at 72°C for 5 min and held at 4°C. After the completion of PCR profile, the products were stored at -20°C until the gel was ready for loading. The amplified PCR products were separated on 1.5% agarose gel electrophoresis. 100bp DNA ladder was also loaded which is used as the molecular marker for determining the product size of primers. Electrophoresis was carried out at 80v for 1 hour and then viewed under Biometra gel documentation unit.

Results and discussion
The daily average temperature for optimal growth of wheat is 22 to 25°C. High temperature above a threshold heat stress) reduces vegetative growth and seed setting in wheat. When plants are subjected to elevated temperature heat shock proteins are induced which has been well reported (Lee et al. 1995; et al., 2007; Ogawa et al. 2007).
HSPs acts as molecular chaperones in maintaining homeostasis of protein folding and play an important role in heat stress (Wang et al. 2004). Based on electrolyte leakage and MSI analysis, HDR 77 was found to be the most susceptible to heat treatment and therefore it was used for gene expression analysis using candidate genes reported in previous studies.

In the present study the expression pattern of HSP80 gene (lane 1 and 2) under normal and late sowing conditions is shown in Fig. 2. Here, HSP80 gene was downregulated under late sowing condition and was highly expressed under normal sowing condition (Fig. 2). 

In our study, the expression of transcription factors like NAC gene, TaVRT-Land WRKY10 were downregulated. This data was in agreement with the previous studies of Busch et al. 2005; Liu et al. 2004; Qi et al. 2004; al. 2007; Qin et al. 2008 The role of WRKY genes in plant defense responses has been well known (Liu et al. 2007). Till now, 74 and 109 members of the WRKY family have been reported in Arabidopsis and rice (Zhang and Wang, 2005; Wu et al. 2005). 

In our study, we reported that these genes (MADSWP12, WRKY72-a, WRKY53b and WRKY72b gene) were upregulated in bread wheat, which were in agreement with previous studies of Qin et al. 2008; and Almeselmani et al. 2012. An interesting study on WRKY genes found that when normally grown leaves of wheat at seedling stage, heading stage and senescing leaves were treated with low temperature, NaCl or PEG treatment, these genes (TaWRKY53-b and TaWRKY72-b) were up-regulated following maturation and leaf senescence. The role of wheat WRKY genes in leaf senescence have been reported (Wu et al. 2008).

TaVRT-1 plays a major role in the regulatory pathway which controls the transition from vegetative to reproductive phase in cereals (Danyluk et al. 2003). In wheat, VRN1/TaVRN1 is responsible for the growth habit and flowering time. TaVRT-1 is a MADS-box transcription factor that is present on the long arm of chromosomes 5. TaVRT-1 is consistently expressed in spring habit plants and is expressed only in winter habit plants after vernalization saturation and results in a switch from vegetative to reproductive growth phase. Danyluk et al. 2003 observed that Spring Norstar has the constitutive expression of TaVRT-1 and Winter Manitou only expresses TaVRT-1 after vernalization saturation. Huang et al. 2015 demonstrated that TaNAC2 plays an essential role in the process of senescence and respond to salt and drought stresses. The function of TaNAC2 was reported in Arabidopsis thaliana (Mao et al. 2012). 

In this study, suppression subtractive cDNA
libraries of wheat produced a fragment of TaNAC2 when treated with polyethylene glycol. Gene expression profiles indicated that TaNAC2 was involved in response to drought, salt, cold and abscisic acid treatment. Overexpression of TaNAC2 resulted in an increased tolerance to drought, salt and freezing stresses in Arabidopsis that were demonstrated by enhanced expression of abiotic stress-response genes. Thus TaNAC2 has a capability for use in transgenic breeding for improving abiotic stress tolerances in crops.

From this study, it is concluded that terminal heat stress results in changes in different parameters like expression of HSPs, RNA signaling, protein synthesis, sugar signaling and carbohydrate metabolism in wheat and is an essential component of thermotolerance. Heat stress responsive genes (HR genes) will help our understanding of molecular basis for heat tolerance in different wheat genotypes and future improvement of heat tolerance in wheat as well as other cereals. From this study the following genes like HSP80, HSP26.6B and WRKY53b have been identified which can be used potentially for further heat stress tolerance studies.in wheat.

Acknowledgment
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References


Mao, X., Zhang, H., Qian, X., Li, A., Zhao, G. and Jing, R. 2012. TaNAC2, a NAC-type wheat transcription factor conferring enhanced multiple abiotic stress tolerances in


Table 1. List of primers used for PCR amplification; Primer 3 software was used to design primer.

<table>
<thead>
<tr>
<th>SL.No.</th>
<th>Primer</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (3'-5')</th>
<th>Annealing temperature (ºC)</th>
<th>Expected product size</th>
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</thead>
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<tr>
<td>1</td>
<td>HSP 80(2)</td>
<td>ATCGTCTCTGACCGTGTTGT</td>
<td>CTGACTTGTCCGTCTTGTTCG</td>
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<td>217</td>
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<td>2</td>
<td>TaVRT-1</td>
<td>ACTGAAGGCGAAGGTTAGA</td>
<td>TGCTTCTCGACGAGTTCTT</td>
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<td>237</td>
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<tr>
<td>3</td>
<td>NAC gene</td>
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<td>GCCACCTTCAATCTTCAGC</td>
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<td>158</td>
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<tr>
<td>4</td>
<td>WRKY72-a</td>
<td>ATGGTGTTGAGTGATGTTG</td>
<td>GTTGTTTCTTCTGAGGCCT</td>
<td>59.5</td>
<td>194</td>
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<tr>
<td>5</td>
<td>WRKY1</td>
<td>GGCTTCTCTTGGATACACT</td>
<td>TTTCCCTATTTTCCTGCGG</td>
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<td>246</td>
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<tr>
<td>6</td>
<td>HSP 26.6B</td>
<td>GTAGCAGCATCGCTTCAAG</td>
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<td>218</td>
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<tr>
<td>7</td>
<td>HSP 26</td>
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<td>GATCAAGCGAGCAGCAACTT</td>
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<td>8</td>
<td>MADS WP 12</td>
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<td>10</td>
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<td>TGGGACAGAGGACCTGACTC</td>
<td>56.0</td>
<td>200</td>
</tr>
</tbody>
</table>

Fig 1. Amplification of actin gene using cDNA isolated from flag leaf of unstressed and heat stressed wheat genotype, HDR77 variety. M represents the marker/ladder 100bp. Lane 1, 3 and 5: normal (unstressed) HDR77; lane 2, 4, 6: terminal heat stress (stressed) HDR77.
Fig 2. Amplification of the HSP 80 and TaVRT 1 gene using cDNA as template isolated from flag leaf of unstressed and heat stressed wheat genotype HDR77 variety. M is the marker of 100bp. Lane 1 and 2: normal and stress condition response of HSP 80 (2 fold change) gene respectively. Lane 3 and 4: normal and stress condition response of TaVRT 1 gene respectively.

Fig 3. Amplification of WPI2 gene and HSP 26.6B gene using cDNA as template isolated from flag leaf of unstressed and heat stressed wheat genotype HDR77 variety. M represents the marker/ladder 100bp. Lane 1 and 2: normal and stress condition response of PISTILLATA-like MADS WPI2 gene (2 fold change) respectively. Lane 3
Fig 4. Amplification of NAC gene and WRKY 53b gene using cDNA as template isolated from flag leaf of unstressed and heat stressed wheat genotype HDR77 variety. M is the marker of 100bp. Lane 1 and 2: normal and stress condition response of NAC gene respectively. Lane A and B: Stress and normal condition response of WRKY 53b gene respectively.
Fig 5. Amplification of certain genes using cDNA isolated from flag leaf of unstressed and heat stressed wheat genotype HDR77 variety. M is the marker of 100bp. Lane 1 and 2: normal and stress condition response of WRKY 72a gene respectively. Lane 3 and 4: normal and stress condition response of WRKY 10 gene respectively. Lane 9 and 10: normal and stress condition response of HSP 26 gene respectively. Lane 13 and 14: stress and normal condition response of WRKY 72b gene respectively.