

Research Article

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

Radha Sharma¹, Jebi Sudan¹, Richa Sharma², Sumita Kumari¹, Romesh K Salgotra¹ and Ravinder Singh^{1*}

¹School of Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Chatha, Jammu, J&K, India-180 009

²Fermentation Technology Division, CSIR- Indian Institute of Integrative Medicine, Jammu

*E-mail : rssandev@gmail.com

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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-quantative 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-quantative 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 hightemperatures. 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 act as molecular chaperones in maintaining homeostasis of protein folding and are related to the acquisition of thermotolerance (Rodríguez *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/primer3-0.4.0).

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 house keeping gene (actin) was also included to investigate 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 semiquantitative 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 (according 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; Lee *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 *HSP 80* gene (lane 1 and 2) under normal and late sowing conditions is shown in Fig. 2 were *HSP 80* gene is less expressed under normal sowing condition. The transcript for *TaVRT- MADS* gene was downregulated under late sowing condition and was highly expressed under normal sowing condition (Fig. 2). Fig. 3 depicts the expression pattern of *MADSWP12* gene under normal and late sowing conditions which shows that this gene is upregulated during stress condition in flag leaf and this gene was less expressed under normal sowing condition. The transcript level for *HSP 26.6B* gene was upregulated during late sowing condition and was less expressed under normal sowing condition. The *NAC* gene (Fig. no. 4) transcripts showed reduced expression levels under stressed conditions when compared to normal conditions. The expression pattern of *NAC* gene under normal and late sowing conditions represent tedthat this gene is downregulated during stress condition in flag leaf and this gene was highly expressed under normal sowing condition.

The expression level of *WRKY53b* gene was upregulated under late sowing conditions compared to normal sowing. The *WRKY53b* gene transcripts showed increased expression levels under stressed conditions when compared to normal conditions. The *WRKY72-a* gene was upregulated during stress condition in flag leaf. The other *WRKY10* gene was upregulated under normal sowing condition. The *HSP26* gene was upregulated under late sowing conditions. The *WRKY72b* gene was upregulated under late sowing when compared to normal sowing conditions (Fig 5). In our study, we reported that the group of genes for heat shock proteins (*HSP80*, *HSP26.6B*, and *HSP26 gene*) were upregulated in bread wheat which is in agreement with previous studies (Wang *et al.* 2004 Qin *et al.* 2008, Almeselmani *et al.* 2012).

Another interesting study on *HSP80-2* cytoplasmic, whose function is in folding has been carried out by Lesage *et al.* 2012 under the research on proteomes of hard and soft near-isogenic wheat lines which revealed that kernel hardness is related to the amplification of a stress response during endosperm development. In yeast *S. cerevisiae*, the molecular chaperone *Hsp26* is one of the component of the heat shock response. Sensing of temperature by *Hsp26* is because of its

middle domain that changes its shape within a narrow temperature range (Franzmann *et al.*, 2008). This in agreement with our results. Wheat grain is a fundamental source of human major food. Wheat varieties in nature are prone to environmental changes. Thus, molecular breeding for high yield has been tried into the wheat chromosome. The results suggested that the acquired genetic traits obtained from rye 2RL enhance resistance to biotic and abiotic stress such as dehydration-induced protein; heat shock protein (*HSP 26.6B*) in wheat-rye translocation lines by altered proteome expression (Kim *et al.* 2011). The role of HSPs as molecular chaperones in maintaining homeostasis of protein folding and its relation in acquisition of thermotolerance in wheat is a well known phenomenon (Wang *et al.* 2004).

In our study, the expression of transcription factors like *NAC gene*, *TaVRT-1* and *WRKY10* were downregulated. This data was in agreement with the previous studies of Busch *et al.* 2005; Liu *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 (*MADSWPI2*, *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 *TaNAC29* 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.

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

Sl.No.	Primer	Forward primer (5'-3')	Reverse primer (3'-5')	Annealing temperature (°C)	Expected product size
1	HSP 80(2)	ATCGTCTCTGACCGTGTGT	CTGACTTGTCGCTCTTGTCG	59.1	217
2	TaVRT-1	ACTGAAGGCCGAAGGTTGAGA	TGCTTCTCGACGAGTTCCTT	59.1	237
3	NAC gene	TCGAAGCGAGTACCTCCATC	GCCACCTTCCAATCTTCGTC	59.5	158
4	WRKY72-a	ATGGTGGTGGTGATGATGGT	GTTGTTGTTCTTGACGGCCT	59.5	194
5	WRKY1	GGCCTCCTCCTTGTAACA	ACTCCGCTCGTACATGTTCA	59	172
6	HSP 26.6B	GTAGCACATCGGCTTCCAAG	TTGTCCCTATTCTCCTGCGC	59.5	246
7	HSP 26	TGCAGGTCCAGTGATGAGTT	GATCAAGCAGCCGAACACTT	59	218
8	MADS WP 12	GCAAGCTCTACGACTTCTGC	GCAGCGAGTTCAGATCTTCG	59.5	195
9	WRKY53-b	AAGTGAGCAACAAGAGCAGC	AGAGCGCTCCACTTCTTCT	59.5	167
10	WRKY 72b	ATGGTGGTGGTGATGATGGT	GTAGCTCCTGGGAAGTTGT	59.5	209
11	Actin	TGGTCGGAATGGGACAGAAG	TGGGACAAGAGGACTGACTC	56.0	200

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.

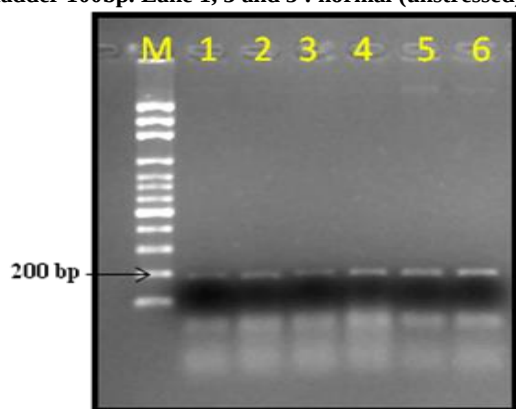


Fig 2. Amplification of the *HSP 80* and *TaVRT 1* gene using cDNA as template isolated from flag leaf of unstressed and heat stressed wheat genotype HDR77 variety M is the marker of 100bp. Lane 1 and 2: normal and stress condition response of *HSP 80* (2 fold change) gene respectively. Lane 3 and 4: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

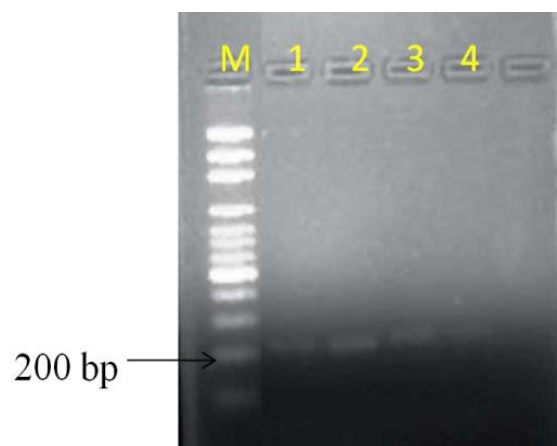


Fig 3. Amplification of *WPI2* gene and *HSP 26.6B* gene using cDNA as template isolated from flag leaf of unstressed and heat stressed wheat genotype HDR77 variety. M represents the marker/ ladder 100bp. Lane 1 and 2: normal and stress condition response of *PISTILLATA-like MADS WPI2* gene (2 fold change) respectively. Lane 3

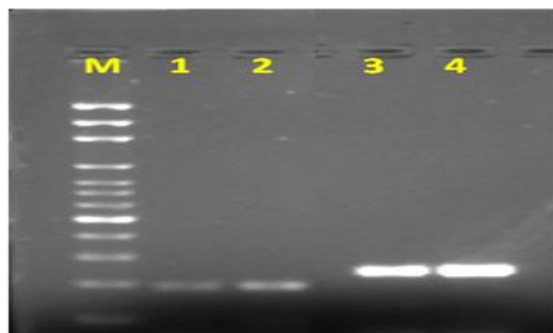


Fig 4. Amplification of *NAC* gene and *WRKY 53b* gene using cDNA as template isolated from flag leaf of unstressed and heat stressed wheat genotype HDR77 variety. M is the marker of 100bp. Lane 1 and 2: normal and stress condition response of *NAC* gene respectively. Lane A and B: Stress and normal 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 *WRKY10* 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.

