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# Expression analysis of stress responsive transcription factors under heat, drought and salinity tolerance in wheat

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**Keywords:**

A Wheat (*Triticum aestivum* L.) is one of the most important staple crops for the global population. It feeds about 40 % of the world population by providing 20 % of total food calories and protein in human diet. It is highly challenged by various abiotic stresses such as drought, salt and heat stress during different growth and developmental stages leading to decreased total production. By altering physiological and metabolic processes, heat, salt and drought stress embarks major threat to the vegetative stage of plant growth and development (Boyer 1982). These stresses continue to be an important challenge to agricultural scientists to develop high performing genotypes. Plants response to heat, salinity and drought is highly negotiated by genotype, growth and developmental stage, duration of exposure, physiological, biochemical and molecular process leading to differential reprogramming of gene expressions (Chaves *et al.,* 2003; Denby and Gehring, 2005; Flowers 2004; Langridge *et al.,* 2006). Among the most important regulatory molecules, DREB family members are central in the regulation of effector molecules. DREB genes, found to be involved in stress signal transduction pathways (Ishitani *et al.,* 1997; Knight *et al.,* 1999; Lee *et al.,* 2001), belong to a large DNA-binding protein family containing a conserved EREBP/AP2 domain (Pandey *et al.,* 2014; Pandey *et al.,* 2016). Different members of this family are able to regulate different processes such as stress response, hormone response and plant development functioning as trans-acting factors in separate signal transduction pathways (Riechmann and Meyerowitz 1998). Similarly, other genes such as NAC, TIR and SCl have also been reported to be involved in modulating the different abiotic stress responses in plants (Puranik *et al.,* 2012).

In this study, semi-quantitative and quantitative PCR technology was used to check the expression behaviour of 5 members of DREB family along with four other abiotic stress responsive genes in two hexaploid wheat genotypes i.e C-306 and HUW 468 at seedling stage exposed to salinity, drought and heat stresses. In this study, semi-quantitative and quantitative PCR technology was used to check the expression behaviour of 5 members of DREB family along with four other abiotic stress responsive genes in two hexaploid wheat genotypes i.e C-306 and HUW 468 at seedling stage exposed to salinity, drought and heat stresses.

Seed of wheat genotypes were surface sterilized with 1% sodium hypochloride for 10 min, rinsed with distilled water for three times and germinated in petri plates coated with moist filter paper at 22°C temperatures under controlled conditions. After five days of germination, seedlings were transferred to the culture bottle filled with full strength Hoagland's solution and allowed to grow for one week. Each genotype was sown in two set each having 3 biological replications. After seven days of growth in Hoagland's solution, one set of seedlings of these genotypes were exposed to osmotic shock temporally by implying 25% (v/v) polyethylene glycol (PEG) 6000 for a time period of 24h while other set was used as untreated control. For heat stress, seedlings were exposed to 42°C for 2 hour under controlled growth chamber, samples from control and stresses seedlings were harvested and immediately frozen in liquid nitrogen and stored at -80°C prior to small RNA isolation.

In this study, based on available literature, we selected a total of 9 allelic variants of DREB genes (*DREB 1A-F 5'-GGCTTGGTTCATTCCCTACC-3' , DREB 1A-R 5'-GCTCTGAGAAGTTGACA CG-3' at Tm 59°C; DREB1B-F 5'-GCCAAAGCACGTGTCAACT-3', DREB1B-R 5'-CAGCGGTTGCCCCATTAG-3' at Tm 60°C; DREB 2A-F 5'-AGGAGGACCTGAGAATACACAA-3', DREB 2A-R 5'-CCTGCTCACCCGATTTGG-3' at Tm 59°C, DREB 2B-F 5'-TACTTGGGGGAAGTGGGTTG-3',*

*DREB2B-R 5'-ATAAGCACGGGCAGCATC-3' at Tm 61°C; DREB 3A-F 5'-CGTGGTGTAAGGCAACGTACT-3', DREB3A-R 5'-CCTCAGCAGTGGGGAACG-3' at Tm 55°C; DREB 3B-F 5'-AGGCTCTGGTTGGGAACG-3', DREB 3B-R 5'-GGAAGTTGGTACGAGCCAGT-3' at Tm 58°C; DREB 4A-F 5'-TACTTGGGGGAAGTGGGTTG-3', DREB 4A-R 5'-GGCGCCATACATTGCTCT-3' at Tm 60°C, DREB 5A-F 5'-TACTTGGGGGAAGTGGGTTG-3', DREB 5A-R 5'-ATAAGCACGGGCAGCATC-3' at Tm 61°C, DREB 5B-F 5'-GAACGTTCCCCACTGCTG-3', DREB 5B-R 5'-GCAGGATGCACAGGGAAGT-3' at* Tm 61°*C) and four abiotic stress responsive genes (NAC-F 5'-CAGGCCATGGTCCAGGTA-3', NAC-R 5'-CACCACTGCCGCCTCTCATG-3'* at Tm 60°*C, TIR-F 5'-GGGCATGTTCTTCCAGGATCG-3', TIR-R 5'- GCAAGAGCCTGAAGAAGCTGG-3' at Tm 58°C, SCL-F 5'-CGCAGGCATCTTCAACTCCAG-3', SCL-R 5'-AACATCGTGGTAAGAACCTAGAG-3'* at Tm 61°*C, Cu protein F 5'-AAACCCAAGCACGTACGCTCG-3', Cu protein R 5'-TCGCCCGCGGCACAAACTAC-3'* at Tm *62°C)* for performing the relative quantification at seedling stage. Primers were designed using PrimerBlast available at (http://www.ncbi.nlm.nih.gov/tools/primer-blast) with a product size of ~200 bp. The β-actin gene was used as a reference/housekeeping gene for data normalization of genes. These primers were then used for analysis of hairpin structure, homo-dimer and heterodimer formation using (http://eu.idtdna.com/calc/analyzer) and primers were made final if they were having ΔG less than ~9 kcal/mol. All the primer sequences were validated using gel electrophoresis of PCR amplicons and by the presence of only single peak on the thermal dissociation (Tm) curve generated by the thermal denaturing protocol, which followed each real time PCR run.

For expression analysis of target transcripts, total RNA was isolated using TRIzol® Reagent (Ambion, USA) following the manufacturer's protocol. The concentration of small RNA and total RNA was determined by Nano-Drop spectrophotometer, ND-1000 (NanoDrop technologies, USA).

cDNA library from total RNA was constructed using Novagen® first strand cDNA synthesis kit (Merck KGaA, Germany) following the user's manual. In short, cDNA library was generated by mixing 1µg of total RNA and 0.5µg of oligo(dT) primer in a 12.5µl reaction volume, incubated at 70°C for 10 min followed by addition of reverse transcriptase 100U M-MuLV Reverse transcriptase, 1µl dNTP mix (10 mM), 2µl of 100 mM DTT and 4µl of 5X buffer in a final reaction volume of 20µl. The reaction mixture was incubated at 37°C for 60 min and stored at -20°C. The concentration and purity of cDNA was quantified using Nano-Drop spectrophotometer, ND-1000 (NanoDrop technologies, USA).

Before proceeding to qPCR, we first checked the amplification of all the genes using routine PCR. The PCR reaction was performed in 20µl reaction volume containing 2µl of cDNA as template, 1µl (10 mM) forward primer, 1µl (10 mM) reverse primer, 1µl of 25 mM $MgCl_2$, 0.25U of Taq DNA polymerase (NEB) and 11.5µl of sterile distilled water (SDW). The PCR protocol was programmed at 94°C for 5 min, then 39 cycles of 94°C for 15s, 60°C for 30s and 72°C for 45s followed by extension of 72°C for 10 min. After PCR confirmation, qPCR was run in a reaction volume of 10µl containing 10ng/µl of cDNA, 5µl of 2X SYBR Green Master Mix (Thermo Scientific), 0.25 µl (3µM) each of forward and reverse primer using above mentioned PCR protocol. β-actin was used as internal control in qRT PCR reaction for data normalization. The reactions were performed in three biological replicates on CFX96™ Real-Time System (Bio-Rad, CA, USA). The threshold cycle (Ct) value of the technical triplicates was averaged and standard deviation was calculated. The relative expression level of all the miRNAs was calculated using the comparative $2^{-\Delta\Delta Ct}$ method. Similarly, semi-quantitative PCR was also performed using cDNA (10ng/µl) used in RT-PCR. PCR amplicons were analysed on 1.5% agarose gel to confirm the amplified products.

*Differential expression behaviour of members of DREB family*

In order to deepen our understanding during heat, drought and salinity stress in two contrasting wheat genotypes i.e. C306 and HUW468, we carried out semi-quantitative and quantitative PCR analysis of nine members of *DREB* gene family. Results indicated that, out of nine members (DREB 1A, 1B, 2A, 2B, 3A, 3B, 4A, 5A, 5B), we could detect only three members (DREB2B, DREB5A and DREB5B) under all the three stress treatment conditions. Semi-quantitative PCR showed varying degree of band intensity of these three genes (Fig 1A). Furthermore, qPCR analysis showed differential expression profile of these genes in both the genotypes under all the three stress treatment conditions (Fig 1B-D) Amongst the three gene, DREB2B was found to be maximally up-regulated followed by DREB5B and DREB5A (Fig 1B-D). DREB2B was upregulated more significantly under drought followed by heat and least under salinity stress (Fig 1B) signifies its active role during drought stress tolerance. Similarly, maximum accumulation of DREB5A and DREB5B was more under heat stress (Fig 1C-D) indicating their active role during heat stress tolerance. The dehydration responsive element

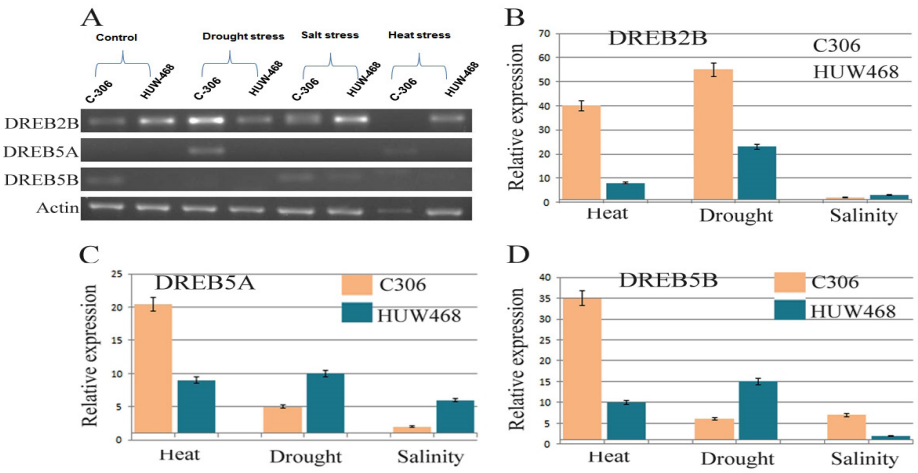


**Fig. 1.** Relative quantification of different members of DREB family in C306 and HUW468 wheat genotypes exposed to heat, drought and salinity stress. A): Semiquantitative PCR; B): DREB2B; C): DREB5A; D): DREB5B. Actin was used as endogenous control for normalizing the Ct value. Data are means of three independent biological replicates ($P < 0.05$, $n = 3$). Error bars represent the means ± SD ($n = 3$).

binding proteins (DREB) are important transcription factors that induce a set of abiotic stress-related genes and impart stress endurance to plants. The DREB transcription factors could be dichotomized as DREB1 involved during low temperature and dehydration signal transduction pathway (Agrawal *et al.,* 2006).. DREB5 is reported for its role during drought and salinity stress response (Mondini *et al.,* 2015). Therefore, differential accumulation of DREB1 and DREB5 might be coordinated with modulating heat, drought and salinity stress tolerance more in C306 than HUW468.

*Differential expression behaviour of other abiotic stress responsive genes:* All the selected abiotic stress responsive proteins showed varying degree of accumulation in both the genotypes under the entire three stress treatment environment (Fig 2A-E). only NAC transcription factors showed higher accumulation in C306 compared to HUW468 under all the three stress condition (Fig 2B) while SCL and Cu protein showed higher accumulation under salinity and heat stress in C306 (Fig. 2D and 2E). Several whole-genome expression profiling studies in *Arabidopsis* and rice have found NAC genes to be induced by at least one type of abiotic stress like salinity, drought, cold or ABA (Fujita, *et al.,* 2004; Kawaura, et al. 2008). Recently, global NAC gene expression analysis in *Arabidopsis* has shown that most of the NAC genes are

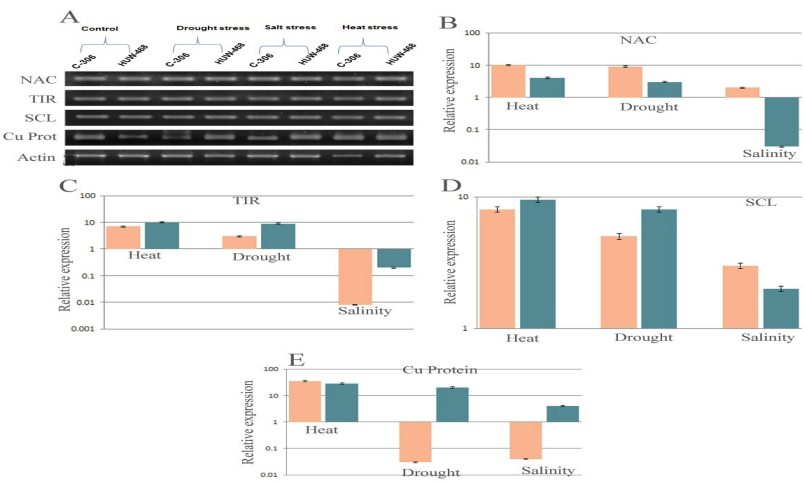


**Fig 2.** Relative quantification of different abiotic responsive genes in C306 and HUW468 wheat genotypes exposed to heat, drought and salinity stress. A): Semi-quantitative PCR; B): NAC; C): TIR; D): SCL; E): Cu Protein. Actin was used as endogenous control for normalizing the Ct value. Data are means of three independent biological replicates ($P < 0.05$, $n = 3$). Error bars represent the means ± SD ($n = 3$).

responsive to salt and extreme temperatures (Jensen, et al. 2010; Zeller, *et al.,* 2009). Up regulation NAC protein in C306 might suggest its active participation in ameliorating drought and salinity as well heat stress tolerance. Similarly, the plant-specific SCL transcription factors play diverse roles in plant development and stress responses. Reports suggested the role of *SCL7* in *Arabidopsis thaliana,* especially with regard to its role in abiotic stress resistance. Expression analysis in poplar revealed that PeSCL7 was induced by drought and high salt stresses (Ma *et al.,* 2010). Our results showed increased expression of SCL under all the three stress condition more significantly in C306 compared to HUW468 which might be linked to its proactive role during these stresses.

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