Insight into the mechanisms of terminal HS-tolerance in wheat mutant with improved nutritional quality through de novo transcriptome sequencing

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Abstract

Terminal HS is one of the main bottle-neck in wheat yield and grain-quality. Here, we have developed wheat mutant (M_3) for HS-tolerance [parent-MP3054- C-306/CB.SPRING BW/CPAN2072 (Parentage)]. To elucidate the mechanism of thermotolerance in mutant, we performed de novo transcriptomic sequencing of mutant (M_3), parent (P_3), and mutant exposed to HS (M_3H). We sequenced 6.5, 7.5, and 7.0 million reads in P_3 , M_3 and M_3H and generated 3,05,537 genes and 5,88,788 transcripts with an N50 of 1,349 bp. We observed 6,120 upregulated and 4,428 downregulated transcripts (M_3 vs P_3), 11,354 upregulated and 12,408 downregulated genes (M_3H vs P_3) and 4817 upregulated and 9085 downregulated genes (M_3H vs M_3). Some of the highly upregulated genes observed were HSP20, SOD, ABC transporters, HSF, etc. and downregulated genes were starch synthase, sucrose synthase, etc. Gene Ontology analysis showed 'ATP-binding' to be most enriched category. Carbon metabolism pathway was observed most altered under HS. We identified 41940 SSRs, 1,10,772 SNPs and 2432 InDels. Potential markers were observed lying on HSP, SOD, STK, and starch synthase. Biochemical markers based characterization showed wheat mutant to be better in HS-tolerance and grain-quality, as compared to parent.

Introduction

Wheat, being considered as staple food grain crop in large part of the world, is nutritionally better, as compared to many other cereals and grains. It is rich in various macro- and micronutrients and provides carbohydrate and proteins in the diet¹. The crop has been reported to be prone to fluctuation in temperature, and even slight variations during critical stages of growth – pollination and grain-filling - causes severe reduction in the yield². Recently, depletion of beneficial nutrients has been observed in wheat crop grown under fluctuating environment, especially during grain-filling³. Terminal HS causes improper fertilization of pollen on stigmatic surface, reduction in pollen tube growth, defunct pollen, synthesis of small starch granules, empty pockets in endospermic tissues, and formation of shrivelled grains⁴. For every 1°C rise in temperature, the yield of wheat has been observed to reduce by 4%⁵. Dubey et al.⁶ simulated the impact of terminal HS on wheat yield and predicted 11.1% decrease in yield by 2050.

Wheat has inherited defence mechanism to protect itself from the adversaries of nature. Terminal HS causes oxidative burst inside the cells which in turn triggered different signalling pathways linked with the modulation of stress-associated genes and proteins⁷. These stress-associated genes (SAGs) and proteins protect the nascent enzymes from denaturation/ aggregation and thus help in maintaining the metabolism of the cells under stress. Wheat, being staple food grain crop, has wide diversities with respect to the HS-tolerance⁸. The variations have been mostly observed in terms of the expression and activities of these SAGs and SAPs present in the cells in response to different environmental conditions.

From last few decades, lots of research work has been targeted towards elucidation of mechanism of thermotolerance in wheat, but with limited success. Even, some of the promising genotypes developed for the thermotolerance have not much been characterized. Very limited information is available on stress-associated genes/ proteins linked with HS-tolerance in public domain⁹. This has masked the potential of various tools and software's involved in decoding the mechanism associated with HS-tolerance.

Omics approaches especially transcriptomics has helped in identification and characterization of novel genes linked with different traits in agriculturally important crops¹⁰. It has helped a lot in decoding the modal plants like Arabidopsis and Brachypodium, etc. and has also helped in characterizing the pathways in crops having complex genome like wheat. With the discovery of next-generation sequencing (NGS) platform, the RNA-seq has been used in a best possible manner to identify the putative transcripts / genes and their Gene Ontology analysis for modelling the functional pathways¹¹. This has led to the identification of candidate genes linked with biotic and abiotic stresses tolerance, and helped in modulating the tolerance level of the plant.

Mutation breeding has been exhaustively used in the past to generate forced variations in the germplasm and development of mutants with desirable traits/ characters. We have many successful stories in the area of mutation breeding especially for

modulating the tolerance level against various stresses. Some of the crops, wherein mutation breeding approach has been used to modulate different traits linked with tolerance are – sorghum¹², Barley¹³, rice¹⁴, wheat¹⁵, etc. Different approaches have been used in the past to develop wheat mutants like using EMS or gamma irradiation in order to develop lines with very high tolerance level. The selected mutants were further used for the back cross breeding program in order to develop genotypes with high tolerance level¹⁶. The generation of desirable donor lines using the approach of mutation has helped the breeder in the development of climate smart crop in the past.

De novo sequencing has potential to identify the SAGs, single nucleotide polymorphism and their localization in the genes responsible for modulating the thermotolerance level in the crop plants¹⁷. The variations in the expression of stress-associated genes in response to varied environmental stresses modulate the tolerance of the plants adapting to adverse environmental conditions¹⁵. Heat stress causes abrupt increase in the expression of heat-responsive transcription factors and SAGs and is important for the adaptation of plant against HS¹⁸. Regulatory and structural genes showed significant variations in the expression under heat stress. The over-expression in response to HS causes accumulation of stress-associated proteins, chaperones such as heat shock proteins (HSPs), and various metabolites¹⁹. Although, identification and annotation of SAGs has been done in exhaustive manner in different crop species, still the number is not sufficient to completely elucidate the mechanism underlying tolerance. Furthermore, isoforms of SAGs are reported to involve in different pathways showing temporal and spatial expression, and to be identified and characterized in order to understand the actual behaviour of different SAGs and SAPs involved in tolerance against different environmental stresses.

De novo transcriptome sequencing is best method for the identification and functional validation of genes and molecular mechanisms underlying different biological processes. It provides a robust option for creating an overview of transcript profiles in different samples under varied treatments in order to understand the mechanism and pathways involved.

In present investigation, a wheat mutant (M4) for HS-tolerance [parent-MP3054- C-306/CB.SPRING BW/CPAN2072 (Parentage)] was developed by Department of Atomic Energy (DAE), Bhabha Atomic Research Centre (BARC), Mumbai. The mutants were further, characterized for their HS-tolerance using RNA-seq in order to identify the possible SAGs, SSRs, SNPs and InDels responsible for modulating the HS-tolerance level of the wheat mutant with improved grain-quality. Even the parents were characterized along with the mutants for the comparative view.

Materials And Methods

Plant materials and treatments

A set of wheat mutants [parent-MP3054- C-306/CB.SPRING BW/CPAN2072 (Parentage)] were created by Department of Atomic Energy, Bhabha Atomic Research Center, Mumbai using the gamma irradiation (dose of 300 Gy) for heat stress-tolerance. The mutant population were screened at different location for thermotolerance and further the seeds of mutants (M4) were used for the molecular and biochemical characterization in order to elucidate the mechanism underlying HS-tolerance. The mutant seeds (in three lots) received from BARC, Mumbai were sown in separate pots (12'x12') inside regulated chambers at National Phytotron Facility, IARI, New Delhi. The seeds were sterilized with 0.1% (w/v) sodium hypochlorite followed by washing with distilled water, and further germination was carried out on moistened sand for 20 days at 22±3 °C. The seedlings with uniform growth were transferred to pot filled with perlite and vermiculite in equal ratio with five seedlings per pot. The pots were kept inside regulated growth chamber under a 16/8 h (day/night) photoperiod, with temperature of 22±3 °C (day-time) and 18±3°C (night-time), and 75% relative humidity. One group was exposed to HS [38±3°C day time and 28±3°C night time] in a sinusoidal mode starting from post-anthesis till harvesting. The samples for the transcriptome were collected during mealy-ripe stage (as per the Feekes scale)²⁰. The samples were collected in triplicates and were further stored at -80°C for further molecular and biochemical research works. The experimental research and field studies on wheat mutant complies with the rules and regulations of Indian Agricultural Research Institute (IARI), New Delhi.

De novo transcriptome sequencing

Total RNA isolation and quality analysis

The total RNA was isolated from the collected samples (root, stem, leaf and spikes) by RNeasy Plant Mini Kit (Qiagen, UK). The quality analysis was performed using the Nanodrop 2000c (ThermoFisher scientific, USA), and Agilent TapeStation. The total RNA having RIN value of more than 7.5 was used for the library preparation. High-quality RNA samples ($RIN \geq 6.5$) were used for the construction of sequencing library after treating it with DNase I (TaKara) in order to remove the DNA contamination. Two biological replicates were used for each experiment.

Construction of RNA-Seq library and Illumina HiSeq4000 sequencing

Transcriptome libraries for sequencing were constructed using the Illumina TruSeq stranded mRNA library construction kit as per the instructions given by the manufacturers (Part # 15008136; Rev. A, Illumina, USA). Briefly, 1 µg of pooled total RNA from the collected samples were used for the isolation of mRNA using oligo dT beads (TruSeq RNA Sample Preparation Kit, Illumina, USA). The purified mRNA was fragmented and reverse transcribed for the synthesis of first strand using random hexamers and Superscript II Reverse transcriptase (Invitrogen, USA). Further, DNA polymerase-I was used for the second strand cDNA synthesis. The cleaned cDNA was ligated with Illumina adapters after end repair. In order to enrich the adapter ligated fragments, the library generated was amplified using PCR. Quality analysis was done using Nanodrop and Bioanalyzer Chip (Agilent Technologies, California, USA). The denatured DNA from the libraries was used for the sequencing using Illumina HiSeq 4000, through the sequencing by synthesis method. The raw sequencing data in Fastq format were retrieved and subjected to quality analysis using SeqQC-V2.0 program (NGS data QC).

De novo assembly and sequence annotation

Pre-processing was carried out by removing the low-quality reads (Q-value < 20), adapter reads and ambiguous reads (>10% 'N' bases) in order to get the clean reads²¹. The clean reads obtained from the parent (P_3), mutant (M_3) and mutant exposed to HS (M_3H) were used for the *de novo* assembly using Trinity v 2.8.4 (<http://trinityrnaseq.github.io>)²². The contigs were clustered, and assembled using Trinity as per the paired-end information. All the assembled transcripts were searched against NCBI nr (NCBI non-redundant protein database). BLAST2GO (<https://www.blast2go.com/>) program was used for the Gene Ontology (GO) annotations of the assembled sequences²³.

GO enrichment analysis and biological pathway characterization

In order to characterise the involvement of assembled transcripts into different pathways in Kyoto Encyclopaedia of Genes and Genomes (KEGG), we have used KEGG Automatic Annotation Server (<http://www.genome.jp/tools/kaas/>). GO enrichment analyses of DEGs were performed using Goatools (<https://github.com/tanghaibao/Goatools>) and KOBAS (<http://kobas.cbi.pku.edu.cn/expression.php>) with GO annotation.

Expression analysis of differentially expressed transcripts (DETs)

The expression level of transcripts was analysed using the FPKM (fragments per kilobase of exon per million mapped fragments) method. RSEM (<http://deweylab.biostat.wisc.edu/rsem/>) was used for analysing the abundance of gene and isoforms. Comparative differential expression analysis was done based on the count of the transcripts in P_3 , M_3 and M_3H using EdgeR v 3.16.5 (Empirical analysis of Digital Gene Expression in R; <http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html>) software ($FDR < 0.05$; $\log_2FC \geq 1$).

Identification of SSRs and variant calling

The assembled transcripts were used for the identification of Simple Sequence Repeats using MicroSatellite identification tool (MISA v 1.0) with default parameters²⁴. In order to identify the polymorphism in the sequence transcripts, SNP calling was performed using Bowtie2 (v.2.2.9) for the - alignment of samples with the assembled transcriptome assembly²⁵. Further, SAMtools v 0.1.19 was used for the variant calling²⁶.

Validation of DE transcripts by quantitative real-time PCR (qPCR)

We randomly selected 20 differentially expressed transcripts (DETs) for the validation using quantitative real time PCR (qRT-PCR). The DETs used for the expression analysis are – Upregulated transcripts - Heat Shock Proteins 70 (TraesCS6B01G367800.1), HSP101 (TraesCS3A01G274400.1), Heat stress transcription factor (TraesCS5D01G393200.1), Calcium dependent protein kinase (TraesCS2D01G207400.1), Serine/threonine-protein kinase (TraesCS2A01G485700.2), WRKY transcriptional factor (TraesCS3D01G173300.2), Peptidyl prolyl isomerase (TraesCS6D01G004600.2), Copper zinc chaperone for superoxide dismutase (TraesCS6B01G362200.1), PsbP-like protein, small HSP17 (TraesCS7A01G161000.1), ABC transporter family (TraesCS3A01G258400.1); downregulated transcripts – Pyruvate decarboxylase (TraesCS2B01G104000.1), Sucrose-phosphate synthase (TraesCS3B01G461800.1), starch synthase (TraesCS2A01G373600.1), Beta-amylase (TraesCS2B01G141700.1), Sucrose transporter (TraesCS6D01G393600.1), Sucrose synthase (TraesCS7D01G036600.2), Zinc transporter (TraesCS2B01G443800.2), Fructose-bisphosphate aldolase (TraesCS7A01G381100.1), Debranching enzyme 1 (TraesCS7B01G034600.1), and ADP-glucopyrophosphorylase (AGPase-LSU). Primers for qPCR analysis were designed manually using the Genefisher2 primer designing software (**supplementary Table S1**). Trizol method was used for the total RNA isolation. We have used 400 ng of DNase-treated RNA for the cDNA synthesis using an oligo (dT) primer with Revert Aid H Minus cDNA synthesis kit (Thermo Fisher Scientific, USA). The expression analysis was performed using Brilliant II SYBR Green qPCR Master mix (Agilent Technologies, USA) according to the manufacturer's instructions. The expression was carried out using the CFX96 platform (BioRad, UK). β -actin (accession no. AF282634) gene was used as endogenous control for normalizing the C_t value. The PCR program and calculation was followed as mentioned in our earlier publication⁷. The relative fold expression was calculated using the Pfaffl method²⁷.

Biochemical characterization of wheat mutant for thermotolerance

Antioxidant enzyme activity assay

The samples (P₃, M₃ and M₃H) were used for the SOD activity assay using the protocol of Beauchamp and Fridovich²⁸. SOD activity was measured by taking absorbance at 560 nm against the reagent blank (phosphate buffer). SOD activity was calculated using the formula: SOD measured = [Control-(Sample-Blank)]/ Control *100; 50% inhibition in O.D. was considered as 1 unit of SOD activity.

Total antioxidant capacity (TAC) analysis

The method of Benzie and Strain²⁹ was used for estimating the total antioxidant potential of collected samples. The absorbance of ferrous coloured form produced from the reduction of ferric-tripyridyltriazine complex was used for calculation of TAC (mM Fe/ g fresh weight).

Accumulation pattern of H₂O₂

The accumulation of H₂O₂ was estimated following the method of Loreto and Velikova³⁰. Leaf samples (0.1 gm) were crushed in liquid N₂ and further 1 mL of Trichloroacetic acid (1%) was added and further centrifuged at 12000 rpm (4°C) for 15 min. Further, a reaction mixture was prepared by adding 0.75 mL of supernatant with 0.75 mL of 10 mM phosphate buffer (pH 7.0) and 1.5 mL of potassium iodide (KI) and the OD was taken at 390 nm. The H₂O₂ concentration was estimated using the extinction coefficient of H₂O₂ (26.6 mM⁻¹ cm⁻¹).

In vivo detection of H₂O₂ inside leaves

The in situ detection of hydrogen peroxide in the leaf was visualized following the protocol, as mentioned by Daudi et al.³¹. In brief, 10 mM Na₂HPO₄ DAB staining solution was prepared by adding DAB (1 mg mL⁻¹), in a separate test-tube with 2.5 mL of 200 mM Na₂HPO₄ and 25 μ L of 0.05% (v/v) Tween 20. We have selected very premature leaves (3 leaves per plant) from parent (P₃), mutant (M₃) and mutant exposed to HS (M₃H) and was treated with 2 mL DAB staining solution in petriplate without exposing to light. For control treatment, we have used 2 mL of 10 mM Na₂HPO₄. The petriplate was kept on standard laboratory

shaker for 4-5 h at 80-100 rpm shaking speed. Further, bleaching solution (ethanol: acetic acid: glycerol = 3:1:1) was added by dispensing the DAB staining solution and was boiled (~90-95°C) for 15 min and the leaf was directly visualized for DAB staining.

Analysis of biochemical traits linked with grain quality

Activity assay of ADP-glucose pyrophosphorylase (AGPase), starch synthase and amylase enzymes

The activity assay of AGPase was estimated using the method of Nivellet et al.³² and Nakamura et al.³³. The developing seeds were ground to fine powder using liq. nitrogen and 100 mg of ground powder was used for the analysis. The conversion of 1 μ M of ADP-glucose into glucose-1-phosphate per min was expressed as one unit enzyme. The activity of SSS was assayed as per the protocol of Nakamura et al.³³. The integrity assay of starch was carried out by analyzing the amylolytic activity in harvested seeds of P₃, M₃ and M₃H. The total amylolytic activity in the grains was estimated as per the modified protocol of Briggs³⁴. In brief, 0.5 g of fine powder was resuspended in 2 mL of ice-cold calcium acetate buffer with pH 6.0 and supernatant was separated through centrifugation. Further, 100 μ L of enzyme extract was added to reaction mixture containing 1 mL of substrate starch solution (1% w/v in reaction buffer) and 1 mL of calcium acetate buffer (pH 4.8) and was incubated at 24 °C for 10 min. The absorbance was read at 620 nm against blank and standard curve of starch was used for the calculation of total amylase activity.

Estimation of Starch Content

The total starch in fractionated sample was estimated as per the protocol of Thayumanavan and Sadasivam³⁵. In brief, the pellet extracted from 0.5 g sample was mixed with 6.5 mL of perchloric acid (52%) and 5.0 mL water followed by incubation at 0°C for 20 min. The supernatant obtained after centrifugation was pooled and the volume was made up to 100 mL. A reaction mixture (RM) was prepared by adding 0.1 mL aliquot, 4 mL anthrone reagent and the final volume was made up to 5 mL followed by heating in a boiling water bath for 8 min. The absorbance was read at 630 nm and glucose standard was used for the estimation of starch by multiplying the value by a factor of 0.9.

Statistical analysis

For de novo transcriptome sequencing, we have used the pooled samples in duplicates, and for molecular and biochemical estimations, we have used three biological replicates. All data analyses were conducted using SPSS Statistics 20.

Results

RNA-seq and de novo transcriptome assembly

Total RNA extracted from the collected samples (leaf, stem, spike and root) were pooled and was used for the RNA-seq using Illumina HiSeq 4000 platform. The raw sequences obtained were subjected to different software's for removing the adaptor sequences, low-quality reads (Q-value < 20) and ambiguous reads (containing more than 10% 'N' bases). The clean reads obtained in different replicates has been presented in supplementary Table S2. We acquired clean reads of ~65031642 (P₃), 80444546 (M₃), and 73850514 (M₃H) with more than 92% Q₃₀ bases. The GC content was ~48% and produced comparable data from the biological replicates. De novo assembly of filtered clean reads were performed using the Trinity v 2.8.4. The preliminary assembly generated 3,05,537 genes and 5,88,788 transcripts with an N50 of 1,349 bp. Among the assembled transcripts, the average contigs length was 836 bp (Table S3). We also predicted the isoforms of the genes predicted with total assembled bases of 18,44,83,645 and average contig length of 603.8 and N50 of 835. The assembled transcripts were plotted against the length of transcripts, as shown in figure 1. The transcript length in the range of 200-250 bp was observed most abundant in the assembled transcripts, followed by transcript length of 1000-1500 bp. GC distribution analysis in the assembled transcripts showed maximum GC percent in the range of 40-45%.

Annotation and classification of *Triticum aestivum* unigenes

All assembled transcripts were subjected to annotation using UniProt Plant Database, gene and protein annotation, organism annotation, gene ontology and pathway annotation. Briefly, the assembled transcriptome sequences were compared with UniProt Plant Database using the NCBI BlastX program with E-value cut-off of 10^{-5} and identity of 40% were retained for further annotation. Overall, we observed 3,62,502 assembled transcripts having at least one significant hit in UniProt Plant Protein Database. The annotation summary for all assembled transcripts is provided in the supplementary Table S4. The complete annotation is divided into the following categories: Total number of assembled transcripts for blastx was 5,88,788 and we observed 3,62,502 hit in uniprot plant database. Overall, the assembled transcripts showed maximum BLASTx similarities to gene sequences from *Triticum aestivum* (~2,20,000 transcripts), *Triticum uratu* (~41,000 transcripts), *Aegilops tauchii* (40,250 transcripts), etc. Other organisms showing maximum hit for the assembled transcripts are - *Brachypodium distachyon*, *Oryza sativa*, *Cicer arietinum*, etc. The top hits of each unigene and the organism name was extracted. The top 15 organisms are shown in figure 2. A summary of the top hit domains identified 1345 heat-responsive transcription factors involved in various biological processes. Similarly, we identified 579, 113 and 22 transcripts showing homology with HSPs, SODs and CDPKs.

Gene ontology (GO) annotation

The functions of the assembled transcripts were classified via GO analysis. The GO terms for all assembled transcripts were extracted wherever possible and BLAST2GO was used for the classification of groups into 3 major categories (supplementary Table S5). The total number of GO terms identified in biological processes, cellular component and molecular function processes are – 3233, 761 and 1963.

In total, assembled transcripts were categorized into 65 functional groups (Fig. 3), and further categorized into three major group i.e. 'biological processes', 'cell component', and 'molecular function'. The dominant sub-categories of the classified genes included 'regulation of transcription' (10,680), 'transcription' (7,760) and 'translation' (5,463) in the 'biological processes' category; 'integral component of membrane' (61,163), 'nucleus' (15,877) and 'cytoplasm' (5,665) in the 'cell component' category; 'ATP binding' (35,127), 'nucleic acid binding' (15,154) and 'protein kinase activity' (12,473) in the 'molecular function' category.

DEGs in wheat mutant (M_3) under HS-treated condition

The expressed transcripts in the P_3 , M_3 , and M_3H were identified using the de novo-assembled transcriptome as a reference. A correlation analysis was carried out based on the FPKM (fragments per kilobase of gene per million mapped reads) values in order to verify the consistency among the samples. We observed very high correlation (Fig. 4; $r > 0.91$) between the replicates of P_3 , M_3 , and M_3H samples, which shows very high consistency in the RNA-seq results. In order to identify the differentially expressed transcripts (DETs), a comparative expression analysis was carried out between P_3 , M_3 and M_3H samples. The number of assembled transcripts considered for differential gene expression analysis was 588,788. DETs were filtered at $\log_2$ fold change cut-off of $+2/-2$ ($p \leq 0.05$). The differential expression analysis of the annotated transcripts was carried out using Cuffdiff program of cufflinks package. $\log_2$ fold change cut-off 2 ($p \leq 0.01$ and 0.05) were separately used as cut-off for upregulated and downregulated genes and isoforms.

On comparative analysis of expression of DEGs in mutant (M_3) over parent (P_3), we observed 6,120 upregulated and 4,428 downregulated transcripts (p -value < 0.01) (Fig. 4a). Similarly, 11,354 upregulated and 12,408 downregulated genes were observed in HS-treated mutant (M_3H) over parent (P_3). The effect of HS on expression of DEGs in mutant showed 4817 upregulated and 9085 downregulated genes (M_3H over M_3). We also predicted the isoforms of identified differentially expressed genes in mutant (M_3) and HS-treated mutant (M_3H) over parents (P_3) (Fig. 4b). We identified 6800 upregulated and 10,120 downregulated transcripts in M_3H vs M_3 ; 7196 upregulated and 7400 downregulated transcripts in M_3 vs P_3 . Similarly, comparative analysis of DETs in mutant vs parent showed 14,228 upregulated and 15,485 downregulated transcripts.

We also plotted top 50 DE genes within each pairwise comparison based on the genes and isoforms. Comparative analysis of DE genes in M_3 and P_3 showed a stretch of transcripts showing upregulation in mutant, as compared to parent and vice versa (Fig. 5a). Similarly, more than 60% of the top 50 DE genes showed upregulation HS-treated mutant, as compared to mutant under control condition. The DE genes which showed upregulation in HS-treated mutant showed downregulation in parent and

vice versa. Expression pattern of isoforms of top 50 DE genes within each pairwise comparison showed significant variations in the expression. Comparative analysis of mutant over parent showed a stretch of DETs showing significant upregulation in mutant and downregulation in parent. Similarly, a stretch of isoforms of DETs were identified in parent showing upregulation and downregulation in mutant (Fig. 5b). Comparative analysis of HS-treated mutant over mutant under control condition showed more than 75% of the isoforms of DETs showing upregulation under HS.

We analysed the average expression between the mean of the normalized counts of the samples used in the respective group through MA plot and volcano plot. The base 2 Logarithm of fold change in expression of M₃H vs M₃ showed much dispersed distribution of upregulated and downregulated transcripts above and below the mean line. Here, we observed more significant number of genes being upregulated, as high number of data points fall above the one threshold on the y-axis. Similarly, large number of genes was observed lying close to the threshold showing non-significant changes in the expression between the M₃H and M₃. We observed similar pattern of expression in M₃ vs P₃, though number of transcripts showing significant downregulation was observed more, as compared to upregulated genes. MA plot of M₃H over P₃ group showed very significant number of genes showing upregulation, as compared to downregulated genes with uniform distribution all over the mean average. We observed large chunk of transcripts lying on the threshold line showing non-significant changes in the Log₂ fold expression in HS-treated mutant (M₃H), as compared to parent (P₃). We also generated the volcano plot analysis of mutant and parent for the visual identification of DEGs with statistically significant variations in the fold expression (Fig. S1).

Functional annotation of DEGs

The DEGs were identified in mutant as compared to parent (M₃ vs P₃), HS-treated mutant, as compared to mutant (M₃H vs M₃) and HS-treated mutant as compared to parent (M₃H vs P₃). Comparative analysis of mutant over parent (M₃ vs P₃) showed 6118 upregulated and 4420 downregulated transcripts (Supplementary Table S6). Some of the highly upregulated transcripts identified were glutamate cysteine ligase (TRINITY_DN6079_c0_g1), Fructokinase-2 (TRINITY_DN9001_c0_g1), 70 kDa HSPs (TRINITY_DN437_c5_g1), Protein disulphide isomerase (TRINITY_DN3156_c1_g2), Kinases (TRINITY_DN68271_c0_g2), Peroxidases (TRINITY_DN9374_c1_g1), ABC transporters (TRINITY_DN60417_c0_g2), ATP synthase subunit alpha (TRINITY_DN7452_c0_g2), Superoxide dismutase [Cu-Zn] (TRINITY_DN17716_c0_g1), HSP20-like chaperone superfamily protein (TRINITY_DN32701_c0_g2), etc. (Table 1) Similarly, some of the downregulated transcripts identified in mutant over parent are - Xylose isomerase (TRINITY_DN19775_c0_g1), Fructose-bisphosphate aldolase (TRINITY_DN15530_c0_g1), Major Facilitator Superfamily protein (TRINITY_DN24775_c1_g1), RNA polymerase II-associated protein 1 (TRINITY_DN16713_c0_g1), Sucrose synthase (TRINITY_DN83591_c0_g1), Starch synthase family protein (TRINITY_DN34859_c0_g1), Sugar transporter (TRINITY_DN25168_c0_g1), UDP-sugar pyrophosphorylase (TRINITY_DN14149_c0_g2), Cysteine protease (TRINITY_DN48681_c0_g1), Zinc transporter (TRINITY_DN31037_c0_g1), etc. (Table 1).

Differentially expressed transcripts (DETs) identified in mutant treated with HS over parent (M₃H vs M₃) showed the presence of 9084 upregulated and 4816 downregulated transcripts (Supplementary table S7). Some of the potential upregulated transcripts identified are - putative chaperone clpb (TRINITY_DN13064_c0_g1), putative heat shock protein (TRINITY_DN21155_c0_g2), Peptidyl prolyl isomerase (TRINITY_DN16896_c0_g1), Calcium-binding EF-hand family protein-like (TRINITY_DN7556_c0_g1), Heat shock transcription factor (TRINITY_DN1205_c0_g1), Universal stress protein family protein (TRINITY_DN3408_c1_g1), Oxidative stress 3 (TRINITY_DN64554_c0_g1), Superoxide dismutase [Cu-Zn] (TRINITY_DN114794_c0_g1), etc. (Table 2). Similarly, some of the identified downregulated DETs are - Cysteine protease (TRINITY_DN14368_c1_g1), Pollen allergen Phl p 5 (TRINITY_DN16967_c0_g1), Late embryogenesis abundant protein (TRINITY_DN42728_c0_g1), L-ascorbate oxidase-like protein (TRINITY_DN4905_c0_g1), Kinase family protein (TRINITY_DN36483_c0_g1), Protein Zinc INDUCED FACILITATOR-LIKE 1 (TRINITY_DN26586_c0_g1), MYB-related transcription factor (TRINITY_DN10260_c0_g3), Pyruvate decarboxylase (TRINITY_DN2112_c1_g3), Alpha/beta-Hydrolases superfamily protein (TRINITY_DN15796_c0_g1), etc. (Table 2).

Further, the DETs were identified by comparing the transcripts of HS-treated mutant over the parent (M₃H vs P₃). We observed 12409 upregulated and 11533 downregulated transcripts in HS-treated mutant, as compared to parent (Supplementary Table S8). Some of the identified upregulated DETs are - Heat shock protein (TRINITY_DN8393_c7_g1), Acid phosphatase 1 (TRINITY_DN18979_c0_g1), ATP-dependent zinc metalloprotease FTSH protein (TRINITY_DN75467_c1_g1), putative chaperone

clpb (TRINITY_DN25343_c0_g1), etc. Similarly, some of the downregulated DETs are - Sucrose synthase (TRINITY_DN4652_c1_g2), Hexosyltransferase (TRINITY_DN55003_c0_g1), Peptide transporter (TRINITY_DN46904_c0_g1), Auxin response factor (TRINITY_DN56713_c0_g1), α -1,4-glucan-protein synthase [UDP-forming] 1 (TRINITY_DN41347_c0_g1), etc. (Table 3).

Overall, we observed maximum transcripts of heat shock proteins showing Log₂ fold expression of 17.0. Similarly, other genes showing high Log₂ fold expression were HSFs, chaperones, peptidyl propyl isomerase, pentatricopeptide repeat-containing protein, universal stress proteins, sucrose phosphate synthase, etc. Similarly some of the downregulated transcripts identified were - Beta-galactosidase, Xylose isomerase, Fructose-bisphosphate aldolase, Protein disulfide-isomerase, Cytochrome P450 family protein, expressed Hexosyltransferase, Pyruvate dehydrogenase E1 component subunit alpha Histone H2B, Gibberellin-regulated protein 2, putative Sugar transporter, Phosphoglycerate mutase-like protein, etc.

Venn diagram analysis

Venn diagram analysis of DETs identified in M₃ vs P₃, M₃H vs M₃ and M₃H vs P₃ has been shown in figure 6. We observed 911 transcripts showing significant (P<0.01) upregulation in mutant, as compared to parent (Fig. 6a). Similarly, 2604 transcripts showed significant upregulation under HS in mutant. Similarly, 4170 transcripts showed significant upregulation in HS-treated mutant (M₃H), as compared to parent (P₃). We observed 2155 common transcripts showing upregulation in M₃H vs M₃ and M₃H vs P₃. Similarly, 5151 transcripts showed significant upregulation in M₃H vs P₃ and M₃ vs P₃. Overall, 57 transcripts showed significant upregulations (P<0.01) in M₃H, M₃ and P₃.

We observed significant (P<0.01) downregulation of 2566 isoforms of DETs in M₃ vs P₃, 1131 transcripts in M₃H vs M₃, and 2729 transcripts in M₃H vs P₃ (Fig. 6b). We observed 1 common transcripts showing significant downregulation in M₃ vs P₃ and M₃H vs M₃. Similarly, 7824 transcripts were observed downregulated in M₃H, as compared to M₃ and P₃; 1726 transcripts showed downregulation in M₃H and M₃, as compared to P₃. Overall, we observed 128 transcripts showing significant (P<0.01) downregulation in HS-treated mutant (M₃H), mutant (M₃) and parent (P₃).

Gene ontology enrichment analysis of DEGs

We have performed GO enrichment analysis using GOATOOLS (Fisher exact test, *P*-value ≤ 0.05) in order to get insight about the functional categories of the DEGs (Klopfenstein et al.³⁶). The total numbers of GO terms identified under different categories are – molecular function (184815), biological processes (100390), and cellular component (114857). The most enriched GO category among these DEGs was 'ATP binding' (35127), followed by 'nucleic acid binding' (15154), 'protein kinase activity', 'serine threonine kinase activity', 'regulation of transcription' (11717), 'translation' (1726), 'DNA integration', 'metabolic process' (17015), 'carbohydrate metabolic process' (4412), 'integral component of membrane' (5717), 'nucleus' (15888), 'chloroplast' (5835), 'cytoplasm' (5776), and 'ribosome' (4808).

Biological pathway annotations

To identify the active biological pathways, functional analysis of proteins, cluster of orthologous groups (COG), orthologous protein-coding genes, protein domains, families and functional sites, the assembled 3,62,502 transcripts were characterized using KEGG, InterPro, RefSeq, eggnoG, OrthoDB, Pfam, PROSITE, PANTHER, and TIGRFAMs. We observed 245 KEGG pathways based on the characterization of 9,815 KEGG-annotated unigenes (Supplementary Table S9). The pathways observed most altered under HS, and in mutant were - 'carbon metabolism', 'ribosome', 'starch and sucrose metabolism', 'biosynthesis of amino acids', photosynthesis, basal transcription factor, DNA replication, and calcium signalling pathways.

Identification of heat-responsive TFs and HSPs in wheat mutant

The annotated data was characterized and searched for the identification of putative HSFs and HSPs in wheat mutant along with their fold expression analysis in parent (P₃), mutant (M₃) and HS-treated mutant (M₃H). We identified 232 HSFs and HSPs showing upregulation in wheat mutant under HS, as compared to control (Supplementary Table S10; Table 4). Heat shock

protein (TRINITY_DN8393_c6_g1) showing homology with wheat *HSP17* (TraesCS4D01G212500.1) showed maximum log fold expression (17.89-fold) in mutant exposed to HS. The transcript was observed lying on chr4D. Similarly, we identified putative HSFs (TRINITY_DN15834_c0_g2) of 1077 bp, which showed 17.3-fold upregulation in M₃H, as compared to control. The TF was observed lying on the chr7A. We also identified three different isoforms of chaperone clpB (TRINITY_DN25343_c0_g1, TRINITY_DN11203_c0_g1, and TRINITY_DN20098_c0_g2) showing 15.5-, 14.7- and 14.4-fold increase in the expression in HS-treated mutant, as compared to control. We observed 39 different isoforms of high molecular weight heat shock proteins especially heat shock 70 kDa protein (TRINITY_DN14477_c0_g1, TRINITY_DN31360_c0_g1, TRINITY_DN6139_c0_g2, TRINITY_DN12920_c1_g1, TRINITY_DN437_c1_g1, TRINITY_DN13029_c0_g2, etc.) showing 11.6-, 9.83-, 8.55-, 8.5-, 8.37-fold upregulation in mutant exposed to HS. Similarly, we identified 6 putative Chaperone proteins DnaJ (TRINITY_DN18040_c0_g3, TRINITY_DN7672_c0_g1, TRINITY_DN23961_c0_g2, TRINITY_DN54525_c0_g1, TRINITY_DN2230_c0_g1, TRINITY_DN2883_c0_g1, TRINITY_DN58791_c0_g1) with digital fold expression of 13.08-, 7.38-, 6.05-, 5.93-, 5.78-, 5.75- and 4.78-fold. Overall, the expression of HSFs and HSPs were observed higher in wheat mutant M₃H. Mutant and parent showed similar pattern of expression of HSFs and HSPs. We observed ~179 putative transcripts of HSPs showing upregulation in wheat mutant, as compared to control. Maximum HSPs were observed lying on chr3D followed by chr3B and chr4B.

Identification of transcripts involved in photosynthesis

We identified 68 transcripts showing homology with genes involved in photosynthesis (Supplementary Table S11). The expression of Photosynthetic NDH sub complex B3 (thylakoid transit peptide) was observed maximum (10.34-fold) in wheat mutant exposed to HS, as compared to control. Similarly, the expression of gene coding for L-ribulose-5-phosphate 3-epimerase (a metalloprotein) and involved in interconversion between D-ribulose 5-phosphate and D-xylulose 5-phosphate was observed highly upregulated (10.27-fold) in M₃H compared with control. We identified 34 transcripts showing homology with photosystem I and II, for example - Photosystem II 10 kDa polypeptide family protein (10-fold), Photosystem II reaction center PsbP family protein (9.73-fold), Photosystem II CP47 reaction centre protein (9.65-fold), Photosystem I P700 chlorophyll a apoprotein A2 (9.31-fold), Photosystem II 10 kDa polypeptide family protein (9.17-fold), etc. We identified 16 putative transcripts of RuBisCo activase involved in conversion of inactive form of RuBisCo into active RuBisCo for further carbon fixation. Ribulose biphosphate carboxylase/oxygenase activase (TRINITY_DN30380_c1_g1) showed 9.61-fold upregulation in wheat mutant, as compared to mutant grown under ambient condition. Other isoforms of RuBisCo activase identified were - TRINITY_DN21223_c0_g1 (7.93-fold), TRINITY_DN30380_c2_g1 (7.58-fold), TRINITY_DN30380_c2_g1 (7.49-fold), TRINITY_DN13450_c0_g1 (7.30-fold), etc. Similarly, we identified 4 sucrose synthase transcripts - TRINITY_DN44228_c0_g1 (4.29-fold), TRINITY_DN50041_c0_g1 (4.17-fold), TRINITY_DN40865_c0_g1 (2.91-fold), and TRINITY_DN92104_c0_g1 (2.84-fold) in wheat mutant.

Mining of transcriptome data for the identification of transcription factors (TFs)

We identified 217 TFs involved in various biological process linked with HS-tolerance in wheat mutant (Supplementary Table S12). Heat shock transcription factor family protein (TRINITY_DN15834_c0_g2) showed maximum expression (log₂ FC 17.3) in M₃H, as compared to M₃ grown under control condition. We identified 36 transcripts showing homology with heat-responsive transcription factors, for example - TRINITY_DN133181_c4_g1 (log₂ FC 13.3), TRINITY_DN301_c0_g1 (log₂ FC 12.9), TRINITY_DN26865_c1_g1 (log₂ FC 11.4), TRINITY_DN139419_c0_g1 (log₂ FC 10.6), TRINITY_DN37726_c2_g1 (log₂ FC 9.95), etc. Basic leucine zipper (bZIP) gene family plays very important role in modulating the defense mechanism of plants against abiotic stress. Here, we identified 4 putative bZIP TFs showing significant upregulation in HS-treated wheat mutant *i.e.* TRINITY_DN38923_c1_g2 (log₂ FC 4.56), TRINITY_DN12902_c0_g4 (log₂ FC 3.57), TRINITY_DN12902_c0_g3 (log₂ FC 3.44), TRINITY_DN2410_c0_g1 (log₂ FC 2.59), TRINITY_DN20899_c0_g1 (log₂ FC 2.5), as compared to mutant under ambient condition. Similarly, MYB transcription factors (TFs) families, is involved in hormone signal transduction, and abiotic stress tolerance, etc. We identified 14 MYB TFs in wheat mutant with significantly higher expression, as compared to parent, for example - TRINITY_DN84781_c0_g1 (log₂ FC 10.34), TRINITY_DN164804_c0_g1 (log₂ FC 8.75), TRINITY_DN72943_c0_g2 (log₂ FC 6.05), TRINITY_DN21219_c1_g1 (log₂ FC 5.79), etc. WRKY transcription factors consist of 60-70 amino acid WRKY protein domains with a conserved motif and a zinc-finger region. Here, we observed 8 putative WRKY TFs showing significant

upregulation in wheat mutant under HS, i.e. TRINITY_DN69699_c0_g1 (log₂ FC 4.08), TRINITY_DN60592_c0_g1 (log₂ FC 2.93), TRINITY_DN35554_c2_g1 (log₂ FC 2.50), TRINITY_DN33330_c0_g1 (log₂ FC 2.43), TRINITY_DN44136_c1_g1 (log₂ FC 2.36), TRINITY_DN3286_c2_g1 (log₂ FC 2.32), TRINITY_DN89270_c0_g1 (log₂ FC 2.15), and TRINITY_DN7487_c0_g1 (log₂ FC 2.13). Overall, we observed significant upregulation of identified heat-responsive TFs in wheat mutant.

Expression of genes involved in stress signalling in wheat mutant

We identified 128 transcripts showing homology with signalling molecule linked with HS-tolerance in wheat mutant M₃H (Supplementary table S13). The expression of signal peptide peptidase-like protein was observed maximum (log₂ FC 12.54) in M₃H compared with M₃. Similarly, sarcoplasmic/endoplasmic reticulum calcium ATPase 3, Calcium-binding EF-hand family protein-like, etc. showed significant upregulation in wheat mutant under HS. CDPK or Calcium-binding EF-hand family protein provides the first line of defense by triggering the signalling mechanism of the plant against HS. We observed 10 transcripts showing homology with CDPK or CBP and has significantly higher expression in wheat mutant under HS, as compared to mutant under control, for example - TRINITY_DN14736_c0_g1 (log₂ FC 9.89), TRINITY_DN14736_c0_g2 (log₂ FC 5.26), TRINITY_DN130772_c0_g3 (log₂ FC 4.64), TRINITY_DN70876_c1_g1 (log₂ FC 4.21), etc. Maximum CDPK/ CBP were observed lying on chr2D. We identified 4 MAP3Ks, 1 MAP2Ks and 2 MAPKs in wheat mutant showing significant upregulation under HS, for example – MAPK (TRINITY_DN62203_c0_g1, TRINITY_DN102354_c2_g1), MAP2Ks (TRINITY_DN37965_c0_g2, TRINITY_DN2937_c0_g1) and MAP3Ks (TRINITY_DN27062_c1_g1, TRINITY_DN27062_c1_g1, TRINITY_DN2937_c0_g1, TRINITY_DN2937_c0_g2).

Validation of DEGs using quantitative Real-Time PCR

In order to validate the accuracy and reproducibility of the RNA-Seq data, we have randomly selected 20 DEGs (10 up-regulated genes and 10 down-regulated genes in the dataset) and further, they were analysed for the expression using qRT-PCR (Fig. 7). The upregulated genes selected for the expression analysis were – calcium dependent protein kinase (*CDPK*), heat-shock protein (*HSP70*), small heat shock protein 17 (*HSP17*), heat-shock protein 101 (*HSP101*), heat shock factor (*HSF*), serine threonine kinase (*STK*), peptidyl-prolyl isomerases (PPIs), superoxide dismutase (*SOD*), oxygen evolving enhancer protein (*OEEP*), and ABC transporter (*ABC-Tra*). The expression analysis of these transcripts in mutant, as compared to parent showed maximum relative fold expression of ABC-Transporter (6.5-fold) followed by serine threonine kinases (*STKs*; 4.28-fold) (Fig. 7a). *HSP17* showed significant increase in the expression (3.74-fold) in M₃, as compared to P₃. Similarly, *SOD* showed 3.9-fold increase in the expression in M₃ compared with P₃.

Expression analysis in mutant exposed to HS showed maximum expression of *HSP17* (15.27-fold) followed by ABC-transporter (*ABC-Tra*; 7.81-fold), as compared to mutant under ambient condition (Fig. 7b). Expression analysis of signalling molecule showed maximum expression of *CDPK* (5.9-fold) in mutant exposed to HS. Similarly, we observed significant increase in the expression of HSPs and antioxidant enzymes in HS-treated mutant, as compared to control. Oxygen evolving enhancer protein (*OEEP*), being important enzyme involved in carbon assimilation showed 2.46-fold increase in the expression in mutant exposed to HS compared to control.

We also validated the differential expression in mutant exposed to HS (M₃H) compared with parent (P₃). The relative fold expression of small *HSP-17* was observed maximum (19.2-fold), as compared to parent (Fig. 7c). Similarly, *ABC-tra* showed 9.4-fold increase in the expression in mutant exposed to HS, as compared to parent. Expression analysis of signalling molecule in mutant exposed to HS showed maximum expression of *CDPK* (5.27-fold) followed by *STK* (3.73-fold). Similarly, superoxide dismutase (*SOD*) showed 4.48-fold and *OEEP* 2.96-fold increase in the expression in HS-treated mutant, as compared to parents. Overall, *HSP17*, *ABC-tra* and *CDPK* showed significantly higher expression in mutant, as compared to parent and might be playing decisive role in modulating the thermotolerance of the mutant.

The downregulated genes selected for the expression analysis were – pyruvate decarboxylase (*PDC*), sucrose phosphate synthase (*SPS*), sucrose synthase (*Suc Syn*), sucrose transporter (*SuT*), soluble starch synthase (*SSS*), rubisco (small subunit; *Rub-S*), β -amylase (β -*Amy*), fructose bis-phosphate aldolase (*FBA*), debranching enzyme (*DBE*), ADP-glucose pyrophosphorylase

(*AGPase*) (Fig. 8). The relative fold expression of *AGPase* was observed minimum (-0.58-fold) followed by *SuT* (-0.62-fold) in mutant, as compared to parent. *RuBisCo* (SSU) showed drastic downregulation in mutant (-0.77-fold), as compared to parents (Fig. 8a). Other genes involved in carbon assimilation especially *SPS*, *Suc Syn*, etc. showed downregulation in mutant; though non-significant variations was observed, as compared to parent.

Expression analysis of M_3H showed maximum downregulation of *Suc Syn* (-0.33-fold) followed by *AGPase* (-0.41-fold) and *SPS* (-0.44-fold), as compared to M_3 under control condition (Fig. 8b). The expression of sucrose transporter (*SUT*) was also observed downregulated (-0.61-fold) in M_3H , which perturbed the transportation of photosynthates from the source (leaves) to the sink (endosperm). Even some of the genes linked with starch biosynthesis pathway like *SSS* and *DBE* showed downregulation in M_3H , as compared to M_3 under control condition. The gene of *RuBisCo* - most abundant enzyme – showed downregulation (-0.77-fold) in M_3H .

Expression analysis of DEGs in M_3 showed maximum downregulation of *Suc Syn* (-4.0-fold) followed by *SPS* (-0.46-fold), as compared to P_3 . Similarly, the genes associated with carbon assimilation like *RuBisCo*, *SPS*, *AGPase*, and *SSS* showed significant downregulation in M_3 , as compared to P_3 . Debranching enzyme, the product of *DBE* showed downregulation (-0.75-fold) in M_3 , as compared to P_3 .

Expression analysis in M_3H showed significant downregulation of sucrose synthase (-0.33-fold) followed by sucrose phosphate synthase (-0.44-fold) and *AGPase* (-0.41-fold), as compared to M_3 grown under control condition. The transcripts especially *RuBisCo*, sucrose transporter, linked with photosynthesis and *SSS*, *DBE*, etc. associated with starch biosynthesis pathway also showed significant downregulation in HS-treated mutant (M_3H), as compared to control. Even catabolic enzyme β -amylase which acts on starch compromising its quality showed downregulation (-0.75-fold) in response to M_3H . Expression analysis in M_3H , as compared to P_3 showed maximum downregulation in sucrose synthase (-0.4-fold) followed by sucrose phosphate synthase (-0.46-fold) and β -amylase (-0.62-fold), as compared to P_3 (Fig. 8c). Even other important genes like pyruvate decarboxylase, *RuBisCo* and *SSS* showed downregulation under HS in mutant compared with parent.

Validation of RNA-seq data in wheat mutant under HS using qPCR

We have randomly selected 10 upregulated and 10 downregulated transcripts from the data-set for the validation of RNA-Seq data using the qPCR. The expression was analysed under ambient and HS-treated conditions. We established a positive correlation between the fold-change values (HS/control) of RNA-Seq and qPCR data ($r^2=0.90$) (Fig. 9). This confirms the reliability of the RNA-Seq data generated and used in present investigation.

Simple Sequence Repeat (SSRs) identified in wheat mutant

We have used the assembled de novo transcriptome data for the identification of putative Simple Sequence Repeats (SSRs) using Perl scripts of MISA (MicroSATellite identification tool). We identified total of 3388 SSRs in Parent (P_3), 4674 SSRs in mutant (M_3) and 33878 SSRs in HS-treated mutant (M_3H). A summary of different markers identified in parent, mutant and HS-treated mutant has been presented in Table 5 and supplementary file 14. We observed abundance of SSRs of trinucleotide type in parent (P_3 – 1740), mutant (P_3 - 2345) and HS-treated mutant (P_3 – 13140).

Variants detected in the DETs involved in modulating thermotolerance in wheat mutant

The assembled de novo transcriptome data were used for the prediction of variants using SAMTOOLS (quality filtered at read depth of 5 and 10; Phred-score ≥ 30). The number of SNPs and InDels identified at RD5 (Read Depth5) and RD10 (Read Depth10) in the P_3 , M_3 and M_3H are presented in Table 6 (supplementary file S15). The assembled transcripts were randomly mapped on to the 21 chromosomes of wheat. SNP calling predicted a total of 1,10,772 intervarietal SNPs at read depth 5 (RD5). We observed significant variations in the coding sequences of identified DETs in mutant over parent. Variant calling were analysed in the transcript sequences by comparing the DETs identified in mutant and parent. We observed potential SNPs in some of the keys DETs such as - Heat shock protein (TRINITY_DN49411_c0_g2_i2) lying on chr3B showed transversion of CC in

parent to CG in mutant. Similarly, SOD (TRINITY_DN5409_c0_g1_i1) used as one of the potential markers for evaluating wheat germplasm for thermotolerance showed A/T allelic transversion in mutant. Serine threonine kinase (TRINITY_DN88200_c0_g3_i1) localized on chr3B is one of the important signalling molecules involved in triggering the defense response of the plants under HS showed C/G allelic transversion. ClpB chaperone – a part of stress induced chaperone system showed transversion of T/G allele. Myb TF, which is involved in hormonal signal transduction and abiotic stress tolerance in plants, showed transition of C/T allele in mutant over parent. bZIP Transcription Factor in mutant. The Basic Leucine Zipper Domain (bZIP domain) protein (TRINITY_DN29470_c0_g1_i3) localized on chr3D has been reported to modulate the tolerance of the plant to the maximum extent, and showed transition of T/C allele in wheat mutant. Universal Stress Protein (TRINITY_DN26896_c0_g1_i3) lying on chr2A showed transition of A/G allele in wheat mutant and has been reported to involved in modulating the stress tolerance of the plants. Similarly, 70 kDa HSP (TRINITY_DN865_c0_g2_i2) localized on chr5D showed transition of C/T allele in mutant. Similarly, Peptidyl prolyl isomerase (TRINITY_DN77799_c0_g1_i1) showed conversion of A/G allele in mutant compared to parent.

In this study, we identified potential gene based InDel markers lying on differentially expressed transcripts identified in wheat mutant and parent. We identified a total of 2432 InDels at read depth of 5 and 1602 InDels at read depth of 10 (supplementary file S15). The InDels identified in parent were 798 (RD5) and 525 (RD10), mutants were 841 (RD5) and 548 (RD10) and mutant exposed to HS were 793 (RD5) and 529 (RD10). Most of the InDels identified were observed lying on predicted genes, signalling molecules, chaperones and genes linked with antioxidant enzymes. For example, protein serine/threonine phosphatase (TRINITY_DN98416_c0_g1_i3) lying on chr3A showed the presence of InDels of alleles in mutant (GTCA/GTCA) and parent (G/G). Similarly, in starch synthase (TRINITY_DN7272_c0_g1_i3), we observed the presence of InDels TGCGC/TGCGC in mutant and TGC/TGC in parent. Being key enzyme of starch biosynthesis pathway, this InDel of SSS was observed lying on chr3A. The protein kinase gene (TRINITY_DN11281_c1_g1_i6) involved in regulating the activity of stress-associated proteins and lying on chr3B showed the presence of InDel GCAGAGTCAG/GCAGAGTCAG in wheat mutant and GCAGAGTCAG/GCAG in parent. Similarly, many InDels were observed lying on serine threonine kinase gene (STKs) which plays very important role as signalling molecule by triggering the defence mechanism against abiotic stress. For example, STK gene (TRINITY_DN12482_c0_g1_i3) lying on chr4B showed the presence of InDels alleles TTGCTGCTGC/TTGCTGCTGC in wheat mutant and TTGCTGCTGC/TTGCTGCTGC in parent.

Analysing the HS-tolerance level and grain-quality of wheat mutant

Characterizing the thermotolerance capacity of wheat mutant

The mutant [parent-MP3054- C-306/CB.SPRING BW/CPAN2072 (Parentage)] developed where further analysed for their HS-tolerance level and grain quality using different biochemical markers. The parent and mutant were grown under ambient condition [$22\pm3^{\circ}\text{C}$ (day time), $18\pm3^{\circ}\text{C}$ (night time)] and HS-treated condition [$38\pm3^{\circ}\text{C}$ (day time), $30\pm3^{\circ}\text{C}$ (night time)]. The flag leaf was used for analysing the tolerance level and the harvested grains were used for the quality analysis. SOD activity profiling in parent and mutant during pollination stage showed maximum activity in HS-treated parent (7.1 U/mg proteins) and minimum in mutant (4.25 U/mg Proteins) under control condition (Fig. 10a). During milky-ripe stage, the SOD activity was observed maximum in HS-treated mutant (6.95 U/mg proteins) and minimum in parent (2.85 U/mg proteins) under control condition. Further, we observed decrease in the SOD activity in both parent and mutant with increase in durations.

In case of guaiacol peroxidase, we observed maximum activity in HS-treated mutant (M_3H) during pollination, milky-ripe and mealy-ripe stages of growth; maximum (63.5 U/mg proteins) was observed during mealy-ripe stage (Fig. 10b). The activity of GPX was observed minimum (10.8 U/mg proteins) in parent under control condition throughout the different stages of growth. Total antioxidant potential is recommended as one of the important biochemical markers for analysing the tolerance level of the plant under stress. Here, we observed significant increase in the TAC in parent (C and HS-treated) from pollination to milky-ripe stage, and further decrease in the TAC was observed during mealy-ripe stage. In case of mutant, we observed decrease in the TAC from pollination to milky-ripe stage, and further significant increase in the TAC was observed during mealy-ripe stage (Fig. 10c). The maximum TAC (16.1 mM/g FW) was observed in HS-treated mutant (M_3H) during mealy-ripe stage.

The accumulation pattern of hydrogen peroxide inside the leaf tissue of parent (P_3), mutant (M_3) and both exposed to HS were visually analysed through NBT assay test (Fig. S2). In case of parent (P_3) under control condition, we couldn't observe any dark patch/spots on the bleached leaves, whereas prominent dark spots were observed in case of parent exposed to HS. Similarly, in case of mutant under control condition, the leaf was slightly darkish showing the minor accumulation of hydrogen peroxide. When the mutant was exposed to HS (M_3H), intense dark blotches appeared in the leaves showing the accumulation of H_2O_2 . Overall, the accumulation was observed maximum in the leaves of mutant exposed to HS.

Characterizing the grain-quality of wheat mutant and parent

The harvested grains were used for analysing the activities of starch synthesizing enzymes (AGPase, SSS), starch degrading enzymes (Amylase) and variations in starch composition under control and HS-treated conditions. In case of wheat parent under control and HS-treated condition, we observed significant ($p<0.05$) decrease in the AGPase activity in response to HS, though non-significant ($p<0.05$) variations were observed in the SSS and amylases activities (Fig. 11a). Similar pattern of decrease in the activities of AGPase, SSS and amylase was observed in case of mutant under HS; though percent decrease was observed less, as compared to parent. The variations in the SSS and amylase activity was observed non-significant ($p<0.05$) in mutant under HS. Overall, AGPase, SSS and amylase present in mutant were observed more thermostable under HS, as compared to parent.

We also characterize the effect of HS on starch composition, especially starch, amylose and amylopectin content in harvested grains of parent and mutant. The starch, amylose and amylopectin content was 78.2, 9.8 and 60.2 % in grains of parent under control condition, which further decreases to 55.1, 7.8, and 51.2 % under HS-treated conditions (Fig. 11b). Grain-quality analysis in mutant showed 75.1, 12.5, and 66 % starch amylose and amylopectin under control condition, which further decreases to 72.4, 10.1, and 58.2 % under HS-treated conditions. In case of parent, we observed ~30% decrease in the starch content under HS, whereas only 4% decrease was observed in mutant. Though, percent decrease was observed non-significant in case of amylose in both parent and mutant. Similarly, we observed 18% decrease in the amylopectin in parent under HS; though percent decrease was observed less (12%) in wheat mutant.

Discussions

Abiotic stresses are detrimental for the normal growth of agriculturally important crops reducing the yield and quality of the grains. The depletion in the grain nutrient has also been observed in most of the crop plants. Wheat is highly sensitive to terminal heat stress and significant yield penalty has been reported in the crop under varied environmental temperature conditions. Different approaches have been used in the past to protect the wheat from terminal HS, but with limited success. Mutation breeding has been used in the past to develop thermotolerant wheat. Here, we have developed wheat mutant [parent-MP3054- C-306/CB.SPRING BW/CPAN2072 (Parentage)] using γ -irradiation. The mutants developed were evaluated for 4 generations and further selected line based on different physiochemical traits linked with HS-tolerance were used for de novo transcriptome sequencing in order to identify SAGs involved in thermotolerance. Through de novo transcriptome of M_3H , M_3 and P_3 , we have assembled 3,05,537 genes and 5,88,788 transcripts with an N50 of 1,349 bp. Mullarkey and Jones³⁷ developed thermotolerant wheat mutants by evaluating 5-d-old seedlings of M2 populations under differential HS. The assembled sequences predicted in present investigation showed maximum similarity with the gene sequences from *Triticum aestivum* (~2,20,000 transcripts). Top hit domain search identified 1345 TFs, 579 HSPs, 113 SODs and 22 CDPKs involved in various processes linked with HS-tolerance. Similarly, Brestic et al.³⁸ developed chlorophyll b-deficient mutant lines (ANK-32A and ANK-32B) and reported less photo-oxidative damage of chloroplast under HS, as compared to wild. Xiong et al.¹⁵ characterized salinity tolerance 1 (st1) mutant of wheat and reported that salinity tolerance was due to mutation in the gene linked with sodium ion transporter.

The characterization of annotated sequences in present investigation based on gene ontology showed maximum transcripts to be involved in regulation of transcription. Kaur et al.³⁹ developed mutants of TaCYPA-1 by substituting sulphur containing amino acids and reported the mutant to be partially thermotolerant. Szalai et al.⁴⁰ reported that wheat mutant with Rht-B1c allele showed better HS-tolerance, as compared to wild-type.

We identified 6,120 upregulated and 4,428 downregulated transcripts (M_3 vs P_3), 11,354 upregulated and 12,408 downregulated genes (M_3H vs P_3) and 4817 upregulated and 9085 downregulated genes (M_3H vs M_3). Pearce et al.⁴¹ characterized the phyB-null and phyC-null mutant and identified 82 DEGs compared with wild-type through RNA-seq and reported their role in flowering and shade-avoidance.

In present investigation, some of the highly upregulated genes identified were HSP20, SOD, HSF, PDI, ABC transporters, etc. Heat shock proteins plays dual role of protecting the nascent proteins from denaturation under HS, and also helps in protein folding in order to make the protein biologically active⁴². SOD has been observed to be at the pivotal position in antioxidant enzymes networking system neutralizing the harmful effect of free oxygen radicals and oxidative burst under HS⁴³. Similarly, ATP-binding cassette transporters (ABC transporters) have been observed to regulate grain formation, other than ATP-powered translocation of many substrates across the membranes⁴⁴. Nirmal et al.⁴⁵ characterized the transcriptome of developing grains of hard (mutant Pin) and soft (wild type) through RNA-seq and identified candidate genes linked with grain hardness.

Ygm (leaf color) mutant of winter wheat was characterized for the identification of candidate gene using RNA-seq and 1227 DEGs with 689 upregulated genes and 538 downregulated genes were identified linked with chlorophyll synthesis and carbon fixation⁴⁶. Similarly, Zhang et al.⁴⁷ studied wheat leaf senescence mutant develop through EMS mutagenesis using RNA-seq and identified 1012 DEGs genes linked with senescence.

Some of the downregulated genes identified in present investigation were - starch synthase, sucrose synthase, sugar transporter, Fru-bis-phosphate aldolase, zinc transporter, LEA protein, pyruvate decarboxylase, auxin responsive factor, etc. Sucrose synthase, being important enzyme involved in photosynthates synthesis, influence the carbon assimilation in plants under HS. The process is also affected by decrease in the activity of sucrose transporter, which affects the distribution process. The downregulation of starch synthase gene affects the starch biosynthesis pathway, especially the filling of the grains⁴.

In present investigation, the most enriched GO category observed was 'ATP binding' (35127), followed by 'nucleic acid binding' (15154), 'protein kinase activity', and 'serine threonine kinase activity'. Similarly, among the pathways - carbon metabolism, followed by ribosome, starch and sucrose metabolism, biosynthesis of amino acids, and photosynthesis was observed most altered. Zhang et al.⁴⁷ performed GO analysis in wheat mutant and reported the importance of TFs and genes linked with protein transporter in leaf senescence. Similarly, Mo et al.¹⁷ identified a wheat mutant line (E529K) involved in suppressing the semi-dwarf phenotype and identified DELLA target genes linked with carbohydrate metabolism.

Here, we identified 128 transcripts linked with signalling molecules and involved in HS-tolerance in wheat mutant. Sun et al.⁴⁸ developed wheat mutant Karcagi 522M7K and observed higher fold expression of TraesCS5A01G543100 gene encoding GA 2- β -dioxygenase through transcriptome sequencing. Wang et al.⁴⁹ worked on wheat stay-green mutant, tasg1 and characterized using RNA-seq and reported that delayed senescence is related to the high N and cytokinin (CK) contents. Similarly, Rikiishi et al.⁵⁰ observed higher expression of TIMING OF CAB EXPRESSION 1 (TOC1) and PHYTOCLOCK 1 homologues in RSD32 (Reduced Seed Dormancy 32) wheat mutant through RNA-seq. Tang et al.⁵¹ developed a novel cleistogamous wheat mutant (ZK001) through static magnetic field treatment and decoded the regulatory mechanisms of cleistogamy through transcriptome analysis. Zhong et al.⁵² identified wheat mutant (SM482gs) having increased grain size and identified some DEGs linked with brassinosteroid biosynthesis pathway through RNA-seq and KEGG analysis.

Here, we identified ~40,000 SSRs, and 1,10,772 SNPs in M_3H , M_3 and P_3 through de novo transcriptome sequencing. Wu et al.⁵³ worked on molecular markers lined with powdery mildew in wheat through de novo transcriptomic approach and identified 283,866 raw SNPs and InDels. Similarly, Kim et al.⁵⁴ developed SNPs linked with salt tolerance through transcriptome sequencing of two Tunisian durum wheat cultivars. Derakhshani et al.⁵⁵ characterized the root transcriptome of wheat and identified 15,207 SNPs and InDels associated with smooth protein function and length of root.

Potential SNPs were observed lying on HSP, SOD, STK, MYB, bZIP, Universal stress protein, and PPLase, which might have changed the thermal stability of these proteins under HS in mutant. These genes have been reported earlier to be potential SAGs

involved in modulating thermotolerance in wheat⁵⁶. We also observed potential InDels lying on starch synthase (prominent enzyme of starch biosynthesis pathway), and STKs (MAPK cascade) providing the structural stability and high activities in wheat mutant under heat stress.

Characterization of wheat mutant, and parent using established biochemical markers linked with thermotolerance showed modulated antioxidant enzymes network in wheat mutant, as compared to parent. Our lab has also reported the pivotal role of Mn-SOD in modulating the HS-tolerance of wheat⁷. Grain quality analysis showed robust enzymes activity in wheat mutant linked with starch biosynthesis pathway; percent reduction in starch synthesis was observed less in mutant, as compared to parent.

Conclusions

To conclude, we have developed stable wheat mutant (M₃) for heat stress tolerance [parent-MP3054- C-306/CB.SPRING BW/CPAN2072 (Parentage)]. In order to understand the mechanism of HS-tolerance, we performed de novo transcriptome sequencing of P₃, M₃ and M₃H and generated 3,05,537 genes and 5,88,788 transcripts with an N50 of 1,349 bp. Maximum similarity of assembled transcripts was observed with *Triticum aestivum* (~2,20,000 transcripts). We observed 6,120 upregulated and 4,428 downregulated transcripts (P₃ vs M₃), 11,354 upregulated and 12,408 downregulated genes (M₃H vs P₃) and 4817 upregulated and 9085 downregulated genes (M₃H vs M₃). We identified 1345 heat-responsive TFs and many other SAGs linked with thermotolerance in wheat mutant. Some of the highly upregulated genes observed were PDI, ABC transporters, HSP20, SOD, HSF, etc. and downregulated genes were Fru-bis-phosphate aldolase, starch synthase, sucrose synthase, sugar transporter, zinc transporter, LEA protein, pyruvate decarboxylase, auxin responsive factor, etc. The most enriched GO category observed was ATP binding, followed by nucleic acid binding. The carbon metabolism pathway was observed most altered under HS. Similarly, we identified total of 41940 SSRs, 1,10,772 intervarietal SNPs (RD5) and 2432 InDels at read depth 5. Potential SNPs and Indels were observed lying on HSP, SOD, STK, MYB, bZIP, Universal stress protein, PPlase, SSS, etc. The developed mutant was observed better in antioxidant defence network and grain-quality, as compared to parent. There is further need to utilize the developed mutant in back-cross breeding program for the development of climate-smart crop.

Declarations

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Author contributions

R.R.K., S.P. conceptualize the idea and plan the experiment; S.B., S.J. developed the wheat mutant using gamma irradiation and evaluated the mutants in different generations; N.J., B.S. performed the genetic and physiological characterization of mutants; R.R.K., S.P. planned and executed the de novo transcriptome sequencing; R.R.K., G.K.R., G.P.S. performed the bioinformatics characterization of generated data; S.H., G.P.S. evaluated the mutant and parent for the tolerance level and grain-quality; K.D., S.P. performed the expression analysis of DEGs and scatter plot analysis; R.R.K., S.B., S.P. drafted the manuscript and edited the language.

Competing interests

The author(s) declare no competing interests.

Availability of data and materials

The data sets generated or analysed during this study are included in this published article and its additional files. All the transcriptome data from 6 samples have been deposited in NCBI's Sequence Read Archive (SRA) under accession number

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Tables

Table 1 List of differentially expressed transcripts (DETs) identified in mutant (M₃), as compared to parent (P₃), the expression was estimated based on FPKM values.

Trinity_GeneID	Wheat_Transcript_ID	Description/Protein Name	Transcript length	Chr	Log FC	P Value	FDR	DEG Type
TRINITY_DN9001_c0_g1	TraesCS7D01G315500.1	Fructokinase-2	1011	chr7D	12.8411	2.14E-43	1.02E-39	UP
TRINITY_DN437_c5_g1	TraesCS6B01G367800.1	70 kDa heat shock protein	1944	chr6B	12.7416	1.36E-42	4.57E-39	UP
TRINITY_DN3156_c1_g2	TraesCS6D01G004600.2	Protein disulfide-isomerase	1017	chr6D	12.0535	1.49E-27	6.54E-25	UP
TRINITY_DN52470_c0_g3	TraesCS5B01G209100.1	Cytochrome P450	1551	chr5B	11.3789	1.02E-26	4.04E-24	UP
TRINITY_DN7452_c0_g2	TraesCSU01G038400.1	ATP synthase subunit alpha, chloroplastic	1017	chrUn	11.3555	2.76E-26	1.01E-23	UP
TRINITY_DN60417_c0_g2	TraesCS7D01G366600.1	ABC transporter family	4554	chr7D	11.1841	1.38E-18	1.50E-16	UP
TRINITY_DN17716_c0_g1	TraesCS6B01G362200.1	Superoxide dismutase [Cu-Zn]	930	chr6B	10.6031	2.18E-13	1.11E-11	UP
TRINITY_DN39705_c0_g1	TraesCS7D01G417400.1	Peroxidase	1038	chr7D	10.5501	3.52E-14	2.01E-12	UP
TRINITY_DN32531_c0_g1	TraesCS7D01G500700.1	Receptor-like kinase	2118	chr7D	10.5036	2.41E-20	3.36E-18	UP
TRINITY_DN27225_c0_g1	TraesCS7D01G409400.1	Kinase family protein	1149	chr7D	10.4169	9.68E-09	2.41E-07	UP
TRINITY_DN77435_c1_g1	TraesCS6B01G011800.1	60 kDa chaperonin	1782	chr6B	10.41	3.49E-16	2.72E-14	UP
TRINITY_DN37189_c1_g1	TraesCS7D01G412400.1	Zinc-transporting ATPase	3012	chr7D	10.0197	6.26E-16	4.71E-14	UP
TRINITY_DN37965_c0_g2	TraesCSU01G133200.1	Mitogen-activated protein kinase kinase	4350	chrUn	10.0037	1.71E-14	1.03E-12	UP
TRINITY_DN36163_c0_g1	TraesCS3B01G107700.1	Signal recognition particle 54 kDa protein	216	chr3B	9.38817	3.02E-11	1.10E-09	UP
TRINITY_DN41999_c3_g1	TraesCS2A01G485700.2	Serine/threonine-protein kinase	2250	chr2A	9.37326	4.89E-13	2.33E-11	UP
TRINITY_DN15316_c0_g1	TraesCS6A01G009200.1	Ferredoxin--NADP reductase	1092	chr6A	9.32158	1.97E-12	8.60E-11	UP
TRINITY_DN75942_c0_g1	TraesCS5A01G217900.1	MYB-related transcription factor	930	chr5A	9.24538	3.01E-11	1.09E-09	UP
TRINITY_DN47321_c1_g1	TraesCS7D01G451700.1	NAC domain-containing protein	939	chr7D	9.24026	6.28E-10	1.87E-08	UP
TRINITY_DN39840_c0_g1	TraesCS2A01G209000.1	Tetratricopeptide repeat (TPR)-like superfamily protein	852	chr2A	9.16031	3.36E-11	1.21E-09	UP
TRINITY_DN24534_c0_g1	TraesCS2A01G432700.1	Calcium-transporting ATPase	3246	chr2A	9.12979	2.15E-11	7.98E-10	UP
TRINITY_DN19775_c0_g1	TraesCS2B01G137300.1	Xylose isomerase	1440	chr2B	-13.8902	3.40E-47	2.57E-43	DOWN
TRINITY_DN15530_c0_g1	TraesCS7A01G381100.1	Fructose-bisphosphate aldolase	1083	chr7A	-12.7118	7.87E-35	9.52E-32	DOWN
TRINITY_DN24775_c1_g1	TraesCS2A01G007500.1	Major Facilitator Superfamily protein, expressed	1707	chr2A	-12.0951	1.99E-20	2.83E-18	DOWN
TRINITY_DN16713_c0_g1	TraesCS7A01G364300.1	RNA polymerase II-associated protein 1	4560	chr7A	-11.6619	2.74E-27	1.18E-24	DOWN
TRINITY_DN32731_c0_g1	TraesCS5A01G411200.1	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	1866	chr5A	-11.494	1.84E-25	5.89E-23	DOWN
TRINITY_DN25168_c0_g1	TraesCS7B01G432900.1	Sugar transporter	1464	chr7B	-11.3452	9.66E-28	4.38E-25	DOWN
TRINITY_DN14149_c0_g2	TraesCS7B01G319900.1	UDP-sugar pyrophosphorylase	1887	chr7B	-10.6663	4.23E-20	5.68E-18	DOWN
TRINITY_DN48681_c0_g1	TraesCSU01G222800.1	Cysteine protease	600	chrUn	-10.119	5.38E-06	8.09E-05	DOWN
TRINITY_DN31037_c0_g1	TraesCS7D01G362100.1	Zinc transporter	1251	chr7D	-10.0291	4.39E-17	3.89E-15	DOWN
TRINITY_DN49096_c0_g1	TraesCS2D01G515800.1	AP2-like ethylene-responsive transcription factor	1461	chr2D	-10.0259	7.01E-16	5.23E-14	DOWN
TRINITY_DN28743_c1_g1	TraesCS7A01G047400.1	Sucrose synthase 2	420	chr7A	-9.92918	4.37E-17	3.89E-15	DOWN
TRINITY_DN29626_c1_g1	TraesCS7D01G551800.1	UDP-glycosyltransferase	1461	chr7D	-9.25463	4.20E-09	1.10E-07	DOWN
TRINITY_DN50322_c0_g3	TraesCS7A01G457700.1	UDP-glucose dehydrogenase 1	885	chr7A	-8.05751	1.13E-05	0.000157	DOWN
TRINITY_DN61962_c0_g1	TraesCS1A01G031300.1	Nitrate transporter 1.1	1749	chr1A	-5.4276	1.70E-07	3.50E-06	DOWN
TRINITY_DN110112_c0_g1	TraesCS7A01G479800.1	Translation initiation factor IF-2, putative	1950	chr7A	-4.33249	0.001151	0.011498	DOWN
TRINITY_DN83591_c0_g1	TraesCS7A01G040900.2	Sucrose synthase	2448	chr7A	-3.24512	0.003796	0.032879	DOWN
TRINITY_DN34859_c0_g1	TraesCS2D01G468900.1	Starch synthase family protein	3999	chr2D	-2.74321	7.38E-05	0.000903	DOWN
TRINITY_DN45564_c1_g1	TraesCS4A01G029000.1	Kinase interacting (KIP1-like) family protein, putative	7143	chr4A	-2.36155	0.004161	0.035624	DOWN
TRINITY_DN60171_c0_g1	TraesCS7B01G034600.1	Debranching enzyme 1	2868	chr7B	-2.23992	2.54E-05	0.00033	DOWN
TRINITY_DN37636_c0_g1	TraesCS7D01G508100.1	Sucrose phosphate synthase	2916	chr7D	-2.15721	2.48E-06	4.06E-05	DOWN

Table 2 List of differentially expressed transcripts (Upregulated and downregulated) identified in wheat mutant exposed to HS (M₃H vs M₃).

Trinity Gene ID	Wheat Transcript ID	Description/Protein Name	Transcript length	Chromosome	Log FC	P-value	FDR	DEG Type
TRINITY_DN13064_c0_g1	TraesCS3A01G274400.1	Chaperone clpb, putative	2742	chr3A	10.56179	2.04E-80	1.67E-75	UP
TRINITY_DN21155_c0_g2	TraesCS4A01G068200.1	Heat shock protein	717	chr4A	15.6719	9.95E-78	4.09E-73	UP
TRINITY_DN126_c1_g1	TraesCS3D01G045700.1	Heat shock protein	456	chr3D	9.813381	1.04E-74	2.85E-70	UP
TRINITY_DN34989_c1_g1	TraesCS2A01G312900.1	Heat-shock protein, putative	660	chr2A	17.46521	2.59E-74	5.33E-70	UP
TRINITY_DN75467_c1_g1	TraesCS3D01G045600.1	Heat shock protein	456	chr3D	12.54418	1.72E-73	2.84E-69	UP
TRINITY_DN16896_c0_g1	TraesCS2B01G294500.1	Peptidylprolyl isomerase	1764	chr2B	15.10596	4.58E-73	6.27E-69	UP
TRINITY_DN7556_c0_g1	TraesCS5D01G269400.1	Calcium-binding EF-hand family protein-like	417	chr5D	8.423285	4.60E-71	5.41E-67	UP
TRINITY_DN11203_c0_g1	TraesCS3D01G273600.2	Chaperone clpb, putative	2688	chr3D	14.71182	7.46E-68	7.67E-64	UP
TRINITY_DN437_c1_g1	TraesCS3D01G351900.1	70 kDa heat shock protein	1959	chr3D	8.500879	8.28E-64	7.57E-60	UP
TRINITY_DN36949_c0_g1	TraesCS4D01G212300.1	Heat shock protein	474	chr4D	8.219156	1.27E-63	1.05E-59	UP
TRINITY_DN20098_c0_g2	TraesCS3D01G273600.1	Chaperone clpb, putative	2742	chr3D	14.47195	3.37E-63	2.52E-59	UP
TRINITY_DN43367_c0_g1	TraesCS3D01G046300.1	Heat shock protein	456	chr3D	8.979644	2.56E-61	1.76E-57	UP
TRINITY_DN21155_c0_g1	TraesCS4B01G225400.1	Heat shock protein	732	chr4B	15.50228	2.91E-61	1.84E-57	UP
TRINITY_DN17739_c0_g1	TraesCS2B01G294500.1	Peptidylprolyl isomerase	1764	chr2B	13.89055	6.55E-55	1.92E-51	UP
TRINITY_DN426_c0_g1	TraesCS5A01G548000.1	BAG family molecular chaperone regulator 6	2523	chr5A	7.162697	4.12E-48	6.51E-45	UP
TRINITY_DN119081_c0_g1	TraesCS2B01G272900.1	Photosystem II 10 kDa polypeptide family protein	786	chr2B	7.044557	1.54E-40	1.28E-37	UP
TRINITY_DN1205_c0_g1	TraesCS5D01G393200.1	Heat shock transcription factor	1134	chr5D	7.761111	4.48E-36	2.41E-33	UP
TRINITY_DN54620_c1_g1	TraesCS3B01G087400.1	Triosephosphate isomerase	762	chr3B	6.805027	3.10E-30	1.05E-27	UP
TRINITY_DN3408_c1_g1	TraesCS4A01G119400.1	Universal stress protein family protein, expressed	510	chr4A	6.044358	2.41E-29	7.75E-27	UP
TRINITY_DN59338_c0_g2	TraesCS7A01G161000.1	10 kDa chaperonin	744	chr7A	9.338163	2.78E-29	8.91E-27	UP
TRINITY_DN16967_c0_g1	TraesCS1D01G390900.1	Cysteine protease	1119	chr1D	-14.9756	9.60E-16	7.47E-14	DOWN
TRINITY_DN14368_c1_g1	TraesCS4A01G000100.1	Pollen allergen Phl p 5	1155	chr4A	-13.8267	2.50E-15	1.85E-13	DOWN
TRINITY_DN42728_c0_g1	TraesCS1D01G231400.1	Late embryogenesis abundant protein	276	chr1D	-12.1853	6.20E-12	2.71E-10	DOWN
TRINITY_DN4905_c0_g1	TraesCS1A01G304100.1	L-ascorbate oxidase-like protein	1680	chr1A	-12.0107	3.54E-09	9.26E-08	DOWN
TRINITY_DN36483_c0_g1	TraesCS4D01G130300.1	Kinase family protein	1203	chr4D	-11.7122	9.18E-13	4.60E-11	DOWN
TRINITY_DN26586_c0_g1	TraesCS4A01G248500.1	Protein ZINC INDUCED FACILITATOR-LIKE 1	1479	chr4A	-11.5167	1.02E-12	5.04E-11	DOWN
TRINITY_DN10260_c0_g3	TraesCS7A01G458700.1	MYB-related transcription factor	1215	chr7A	-11.3864	1.55E-09	4.36E-08	DOWN
TRINITY_DN2112_c1_g3	TraesCS2B01G104000.1	Pyruvate decarboxylase	1446	chr2B	-10.4472	9.02E-06	0.000104	DOWN
TRINITY_DN15796_c0_g1	TraesCS5A01G157600.2	Alpha/beta-Hydrolases superfamily protein	900	chr5A	-10.1508	9.63E-07	1.43E-05	DOWN
TRINITY_DN34867_c0_g1	TraesCS2A01G411600.1	14 kDa proline-rich protein DC2.15	381	chr2A	-9.34396	4.34E-06	5.43E-05	DOWN
TRINITY_DN58916_c1_g1	TraesCS2B01G165700.1	Basic-leucine zipper (BZIP) transcription factor	924	chr2B	-9.3152	3.25E-09	8.55E-08	DOWN
TRINITY_DN109916_c0_g1	TraesCS5A01G345500.1	family protein, putative SAUR-like auxin-responsive protein family	411	chr5A	-9.08037	7.38E-08	1.45E-06	DOWN
TRINITY_DN10664_c1_g1	TraesCS3B01G461800.1	Sucrose-phosphate synthase	3210	chr3B	-8.68248	8.37E-07	1.26E-05	DOWN
TRINITY_DN34916_c0_g1	TraesCS6D01G088000.1	Cation-transporting ATPase 4	1686	chr6D	-8.24959	1.63E-06	2.28E-05	DOWN
TRINITY_DN70922_c0_g1	TraesCS2A01G373600.1	Starch synthase, chloroplastic/amyloplastic	1800	chr2A	-8.1863	2.23E-06	3.01E-05	DOWN
TRINITY_DN29846_c1_g1	TraesCS7B01G270400.1	Solute carrier family 40 member 1	1491	chr7B	-7.85494	1.05E-05	0.000119	DOWN
TRINITY_DN7247_c0_g1	TraesCSU01G109000.1	UTP--glucose-1-phosphate uridylyltransferase	1404	chrUn	-7.71509	7.06E-14	4.21E-12	DOWN
TRINITY_DN4814_c0_g1	TraesCS3A01G131900.2	Basic-leucine zipper (bZIP) transcription factor family protein	921	chr3A	-5.90508	9.93E-09	2.38E-07	DOWN

TRINITY_DN2096_c0_g1	TraesCS7B01G437000.1	TNF receptor-associated factor family protein	1065	chr7B	-5.43817	6.65E-10	2.01E-08	DOWN
TRINITY_DN9454_c2_g1	TraesCS2B01G141700.1	Beta-amylase	1584	chr2B	-5.21042	0.000126	0.001105	DOWN

Table 3 List of differentially expressed transcripts (Upregulated and downregulated) identified in wheat mutant exposed to HS, as compared to parent (M₃H vs P₃), the expression was estimated based on FPKM values.

Trinity Gene ID	Wheat Transcript ID	Description/ Protein Name	Transcript length	Chromosome	Log FC	P-value	FDR	DEG
TRINITY_DN8393_c7_g1	TraesCS5B01G245700.1	Heat shock protein	465	chr5B	17.4165	4.84E-48	2.26E-44	UP
TRINITY_DN18979_c0_g1	TraesCS7A01G040400.1	Acid phosphatase 1	843	chr7A	16.763	1.01E-45	2.90E-42	UP
TRINITY_DN75467_c1_g1	TraesCS3D01G045600.1	Heat shock protein	456	chr3D	16.2029	1.73E-62	1.53E-57	UP
TRINITY_DN4686_c2_g1	TraesCS7A01G188300.1	ATP-dependent zinc metalloprotease FTSH protein	2055	chr7A	16.0704	2.22E-24	3.82E-22	UP
TRINITY_DN21155_c0_g2	TraesCS4A01G068200.1	Heat shock protein	717	chr4A	15.5748	1.63E-61	7.25E-57	UP
TRINITY_DN25343_c0_g1	TraesCS3A01G274400.1	Chaperone clpb, putative	2742	chr3A	15.413	7.66E-47	2.72E-43	UP
TRINITY_DN21155_c0_g1	TraesCS4B01G225400.1	Heat shock protein	732	chr4B	15.4041	1.25E-50	8.56E-47	UP
TRINITY_DN31360_c0_g1	TraesCS3B01G390700.1	70 kDa heat shock protein	1959	chr3B	13.2798	3.11E-58	6.90E-54	UP
TRINITY_DN3333_c1_g1	TraesCS2B01G272900.1	Photosystem II 10 kDa polypeptide family protein	786	chr2B	4.70889	2.07E-05	0.00013	UP
TRINITY_DN48827_c0_g1	TraesCS6A01G310500.1	Peroxidase	408	chr6A	4.67811	2.76E-06	2.15E-05	UP
TRINITY_DN111015_c0_g3	TraesCS2D01G033200.2	Heat shock protein 90	2139	chr2D	4.67166	4.95E-06	3.60E-05	UP
TRINITY_DN69574_c0_g3	TraesCS6B01G143000.1	Calcium-dependent lipid-binding domain protein	888	chr6B	4.64715	5.52E-10	1.05E-08	UP
TRINITY_DN4652_c1_g2	TraesCS7D01G036600	Sucrose synthase	2448	chr7D	-2.234	0.047784	0.11515	DOWN
TRINITY_DN55003_c0_g1	TraesCS2A01G103200	Hexosyltransferase	1884	chr2A	-2.795	0.04798	0.11554	DOWN
TRINITY_DN46904_c0_g1	TraesCS3B01G424800	Peptide transporter	1656	chr3B	-2.94	0.048051	0.11562	DOWN
TRINITY_DN56713_c0_g1	TraesCS7A01G416400	Auxin response factor	3522	chr7A	-2.818	0.045529	0.11089	DOWN
TRINITY_DN41347_c0_g1	TraesCS2A01G174400	Alpha-1,4-glucan-protein synthase [UDP-forming] 1	1290	chr2A	-2.034	0.042749	0.10547	DOWN
TRINITY_DN32011_c2_g1	TraesCS5D01G017000	3-ketoacyl-CoA synthase	1419	chr5D	-3.832	4.19E-06	3.10E-05	DOWN
TRINITY_DN58088_c0_g1	TraesCS6D01G023900	Mitochondrial transcription termination factor-like	1170	chr6D	-7.867	4.06E-06	3.02E-05	DOWN
TRINITY_DN54835_c0_g1	TraesCS7D01G425000	TOPLESS-related 2	1200	chr7D	-3.002	4.06E-06	3.02E-05	DOWN
TRINITY_DN176814_c0_g1	TraesCS5A01G022800	F-box family protein	1545	chr5A	-7.725	4.06E-06	3.02E-05	DOWN
TRINITY_DN57550_c0_g2	TraesCS2D01G043500	Cytochrome P450 family protein	1707	chr2D	-7.725	4.06E-06	3.02E-05	DOWN
TRINITY_DN72164_c0_g1	TraesCS3A01G413500	P-loop containing NTP hydrolases superfamily protein	234	chr3A	-7.729	3.92E-06	2.94E-05	DOWN
TRINITY_DN77863_c0_g2	TraesCS7D01G412800	Zinc-transporting ATPase	2838	chr7D	-7.729	3.92E-06	2.94E-05	DOWN
TRINITY_DN72746_c0_g1	TraesCS4A01G259900	Glycosyltransferase	1368	chr4A	-8.366	3.78E-06	2.84E-05	DOWN
TRINITY_DN39612_c0_g2	TraesCS6D01G245300.1	Growth-regulating factor	1230	chr6D	-5.549	3.20E-06	2.45E-05	DOWN
TRINITY_DN36433_c0_g1	TraesCS1B01G340700.1	Signal recognition particle 54 kDa protein	1485	chr1B	-8.046	3.22E-06	2.46E-05	DOWN

Table 4 List of differentially expressed transcripts (DETs) showing maximum homology with heat shock transcription factors (HSFs) and heat shock proteins (HSPs) in wheat mutant (M₃) and parent (P₃) through de novo transcriptomic approach.

Description/Protein Name	Trinity Gene ID	Wheat Transcript ID	Transcript length	Chromosome	Log ₂ FC	FDR
Heat-shock protein	TRINITY_DN8393_c6_g1	TraesCS4D01G212500.1	468	chr4D	17.8942	5.56E-36
Heat shock protein	TRINITY_DN8393_c7_g1	TraesCS5B01G245700.1	465	chr5B	17.5158	1.72E-52
Heat-shock protein, putative	TRINITY_DN34989_c1_g1	TraesCS2A01G312900.1	660	chr2A	17.4652	5.33E-70
Heat shock protein	TRINITY_DN89874_c0_g1	TraesCS3A01G034500.1	456	chr3A	17.4595	1.17E-42
Heat shock TF family protein	TRINITY_DN15834_c0_g2	TraesCS7A01G360400.1	1077	chr7A	17.3232	9.55E-36
Heat shock protein	TRINITY_DN21155_c0_g2	TraesCS4A01G068200.1	717	chr4A	15.6719	4.09E-73
Chaperone clpb, putative	TRINITY_DN25343_c0_g1	TraesCS3A01G274400.1	2742	chr3A	15.5077	8.15E-54
Heat shock protein	TRINITY_DN21155_c0_g1	TraesCS4B01G225400.1	732	chr4B	15.5023	1.84E-57
Heat shock protein	TRINITY_DN77260_c0_g1	TraesCS4B01G225400.1	732	chr4B	14.7216	5.86E-48
Chaperone clpb, putative	TRINITY_DN11203_c0_g1	TraesCS3D01G273600.2	2688	chr3D	14.7118	7.67E-64
Heat shock protein	TRINITY_DN77260_c1_g1	TraesCS4D01G226000.1	717	chr4D	14.7054	4.46E-40
Chaperone clpb, putative	TRINITY_DN20098_c0_g2	TraesCS3D01G273600.1	2742	chr3D	14.4719	2.52E-59
Heat shock transcription factor	TRINITY_DN26865_c3_g1	TraesCS7B01G267300	1062	chr7B	14.4156	4.26E-17
Heat shock protein	TRINITY_DN1603_c1_g1	TraesCS3D01G046700	456	chr3D	14.3447	4.70E-46
Heat-shock protein, putative	TRINITY_DN835_c0_g1	TraesCS4D01G213100	474	chr4D	13.4788	1.39E-44
Heat shock transcription factor	TRINITY_DN133181_c4_g1	TraesCS5A01G383800	1119	chr5A	13.361	6.59E-50
ATP-dependent chaperone ClpB	TRINITY_DN15566_c0_g1	TraesCS4B01G380800	2928	chr4B	13.2117	2.76E-49
Heat shock protein	TRINITY_DN154892_c0_g1	TraesCS3B01G130400	483	chr3B	13.2036	1.82E-16
Chaperone protein DnaJ	TRINITY_DN18040_c0_g3	TraesCS3B01G247200	576	chr3B	13.0834	1.24E-18
Heat shock 70 kDa protein	TRINITY_DN14477_c0_g1	TraesCS4B01G243400	1743	chr4B	13.0595	2.19E-47
Heat shock transcription factor	TRINITY_DN301_c0_g1	TraesCS7B01G267300	1062	chr7B	12.9879	1.51E-41
Heat-shock protein, putative	TRINITY_DN4941_c0_g1	TraesCS2A01G312900	660	chr2A	12.9878	1.55E-49

Table 5 List of Simple Sequence Repeats (SSRs) identified in heat shock treated wheat mutant (M₃H) and parent (P₃) through mining of de novo transcriptome data using MicroSATellite identification tool (MISA v 1.0).

Samples	P1	P2	P3	P4	P5	P6	C	C*
P3-R1	611	707	1740	124	17	9	165	15
P3-R2	518	607	1582	115	14	9	151	10
M3-R1	896	954	2345	167	28	16	255	13
M3-R2	653	727	1764	126	17	9	186	15
M3HS-R1	6129	5981	13140	864	157	79	1273	31
M3HS-R2	3419	3359	8010	537	90	48	733	17

Table 6 Prediction of Single Nucleotide Polymorphisms (SNPs) and insertion-deletion mutations (InDels) in wheat mutant (M₃) and parent (P₃) through mining of de novo transcriptome data using SAMTOOLS.

	Variants			
	SNPs		INDELs	
Samples	RD5	RD10	RD5	RD10
Parent	32050	25012	798	525
Mutant	38020	26570	841	548
Mutant+HS	40702	27580	793	529
Total	110772	79162	2432	1602

*RD-Read depth

Figures

Figure 1

Mapping of assembled transcripts against the length of transcripts: the length of transcripts has been presented in the uniform range.

Figure 2

Top hits analysis of assembled transcripts with the organism: the top 15 organisms showing maximum similarity with the assembled transcripts were identified.

Figure 3

Gene Ontology analysis of assembled transcripts: the identified transcripts were categorized into 65 functional groups grouped under three major groups - biological processes, cell component, and molecular function.

Figure 4

Differentially expressed transcripts and isoforms identified in P3, M3 and M3H: (a) the comparative expression analysis of DETs between the samples, (b) comparative expression analysis of isoforms of DETs between the samples, expression analysis was based on the FPKM (fragments per kilobase of gene per million mapped reads), P3 – parent, M3 - mutant, M3H – mutant exposed to HS.

Figure 5

Expression of top 50 DE genes within each pairwise comparison based on the genes and isoforms: (a) Expression pattern of top 50 DE genes identified in wheat mutant, as compared to parent, (b) Expression pattern of isoforms of top 50 DE genes.

Figure 6

Venn diagram analysis of DETs identified in wheat mutant: (a) DETs identified in mutant under ambient and HS-treated conditions were plotted against parent, (b) Isoforms of DETs identified in wheat mutant.

Figure 7

Validation of randomly selected upregulated transcripts using qRT-PCR: (a) expression analysis of upregulated transcripts in mutant, as compared to parent, (b) expression analysis of upregulated transcripts in mutant exposed to HS, as compared to mutant under control condition, (c) expression analysis of upregulated transcripts in mutant exposed to HS, as compared to parent.

Figure 8

Validation of randomly selected downregulated transcripts using qRT-PCR: (a) expression analysis of downregulated transcripts in mutant, as compared to parent, (b) expression analysis of downregulated transcripts in mutant exposed to HS, as compared to mutant under control condition, (c) expression analysis of downregulated transcripts in mutant exposed to HS, as compared to parent.

Figure 9

Validation of RNA-seq data using qPCR: scatter plot analysis was carried out using the expression of ten DETs obtained through RNA-seq and qRT-PCR in wheat mutant.

Figure 10

Characterizing the HS-tolerance level in wheat mutant and parent: SOD activity in parent and mutant under control and HS-treated conditions at different stages of growth, (b) GPX activity in parent and mutant under control and HS-treated conditions at different stages of growth, (c) TAC in parent and mutant under control and HS-treated conditions at different stages of growth.

Figure 11

Characterizing the variations in the grain-quality of wheat mutant and parent: (a) activity assay of starch synthesizing and degrading enzymes in wheat parent and mutant under control and HS-treated conditions, (b) starch composition analysis in wheat parent and mutant under control and HS-treated conditions.

Supplementary Files

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- TableS9.xlsx
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