Identification of novel starch synthase genes using *de novo* assembly and heat-induced expression and activity in developing wheat (*Triticum aestivum* L.)

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Terminal heat stress (HS) reduces quantity and quality of the grains. Wheat is highly sensitive to HS and a wide diversity has been observed among different genotypes for HS-tolerance. The enzymes associated with starch biosynthesis are severely affected by HS, especially starch synthases (SS). Different isoforms of SS have been reported from the rice, maize etc.; however, limited information is available in the wheat. In this study, we investigated the effect of HS on contrasting wheat (*Triticum aestivum* L.) cultivars — HD2985 (thermotolerant) and HD2329 (thermosusceptible) at grain-filling stage. Here we report the identification and cloning of three putative SS genes from wheat. The putative SS has been characterized *in silico* with other SS reported from different plant and non-plant sources in order to investigate potential functional and evolutionary relationships between SSs. The correlation between HS and SS activity and other biochemical parameters associated with thermotolerance in two cultivars was also studied. Three putative SS genes (transcript_7326, transcript_24546 and transcript_38472) were cloned from HD2985 cultivar under HS by data mining generated from whole transcriptome analysis. The sequences were *in silico* characterized and submitted in NCBI GenBank (accession nos. KM20614 3,KM206144 and KJ854903). Glucosyltransferase domain was observed in all the three sequences. Based on ClustalW alignment, SSs were classified into four distinct families. Expression analysis and SS activity assay showed significantly higher transcript level and SS activity during mealy-ripe stage than at milky-ripe stage in HS-treated HD2985, compared to HD2329 cultivar. Both the cultivars showed decrease in starch content under HS and the overall content was higher in HD2985, compared to HD2329. Similarly, guaiacol peroxidase, catalase, free amino acid and total antioxidant capacity were higher during mealy-ripe stage in HD2985. HS during milky-ripe had more severe impact on the overall physiochemical properties of wheat grain and thus needed to be targeted using different approaches in order to enhance the quality and yield of wheat under HS.

Keywords: *Triticum aestivum* L., Transcript, Heat stress, Starch synthase, qRT-PCR, Starch, Wheat, Catalase, Peroxidase, Total antioxidant capacity.

Wheat (*Triticum aestivum* L.) is staple cereal grain crop widely cultivated in large areas of the world. It provides 12% of the carbohydrate and 40% of the calorie in the diet. A decrease in the wheat yield as well as quality of grains has been reported from different parts of the world because of change in the global temperature. Wheat is highly sensitive to heat stress (HS), especially during critical growth stages, such as flowering and grain filling which adversely affect the yield. HS causes pollen sterility, drying of stigmatic surface, incomplete fertilization, disturbed

carbon assimilatory pathways, denaturation /aggregation of the enzymes, defragmented starch granule synthesis etc[1]. The yield losses can be in the order of 190 kgha[-1] for every 1°C rise in average temperature and in some situations even having a more severe effect in yield loss[2]. Elevation in temperature during grain-filling stage in wheat has been reported to severely affect the starch biosynthesis pathway, especially the enzymes, reducing their activity and hence causing empty pockets in endospermic tissues and shriveled grain formation[1].

Starch, being biopolymer, is an important source of carbon and energy. It is a major component of wheat endosperm that accounts for 65-75% of the dry weight of the mature grain and is highly related to end-use quality of wheat-based products[3,4]. A series of biosynthetic enzymes are involved in starch synthesis, such as ADP-glucopyrophosphorylase (AGPase), starch synthase (SS), branching enzyme (BE) and

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Abbreviations: CAT, catalase; FAA, free amino acid; GPX, guaiacol peroxidase; GBSS, granule bound starch synthase; HS, heat stress; qRT-PCR, quantitative real-time PCR; SS, starch synthase; TAC; total antioxidant capacity.

debranching enzyme (DBE). Multiple isoforms of each enzyme are present in higher plants. Starch is made up of two α-1,4-glucan polymers; amylose is the smaller almost linear polymer having 0.5% α-1,6 linkages with a degree of polymerization of 1,000 to 5,000 glucose units, while amylopectin is larger and the frequency of α-1,6-glycosidic linkages is higher between the glucan chains. Wheat starch generally contains A and B-types starch granules having different effects on starch properties and end-use qualities.

Starch synthases (SS; EC 2.4.1.21 and EC 2.4.1.242) are involved in elongating the glucan chains of amylose and amylopectin. SS catalyzes the transfer of glucose moiety to the non-reducing end of a pre-existing glucan chain. So far, five SS classes i.e. SS-I[5], -II[6], -III[7-9], -IV and granule bound SSI (GBSSI)[10] have been identified from different plant sources. In cereals, maize has been exhaustively studied for SS enzymes, wherein other than GBSS, genes of SSI, SSIIa and SSIIb have been identified and cloned[9].

Duplication of SS encoding genes gives rise to different isoforms[11]. SSI and SSII elongate the short and intermediate chains, whereas SSIII and GBSSI elongate the long chains of amylopectin, respectively[12]. GBSSI has been reported to be involved in making amylose[13]. Recently reported SSIV isoform has been observed to involve in starch granule initiation in *Arabidopsis*[14]. SS isoforms are reported to have very unique role in starch biosynthesis pathway; functional overlap has been observed in some of the SS transgenic and mutant lines[14]. They have been reported from different plastidial locations. GBSSI is starch granule-localized, whereas SSIII and -IV are exclusively localized in the stroma[14], SSI and -II are localized between the stroma and granule. The kinetic behaviors of different SS isoforms depend on their localization and interaction with the substrate which influences the granule structure[10]. SS, especially reported from wheat is heat-labile, whereas thermostable SS has been observed in maize and sorghum[5].

Here we report the identification and cloning of three putative SS genes from wheat. The putative SS has been characterized in silico with other SS reported from different plant and non-plant sources in order to investigate potential functional and evolutionary relationships between SSs. The correlation between HS and SS activity and other biochemical parameters associated with thermotolerance has also been studied in contrasting wheat cultivars — HD2985 (thermotolerant) and HD2329 (thermosusceptible).

Materials and Methods
Sowing and stress treatments

Pre-treated seeds (Bavistin @ 0.25%) of HD2985 and HD2329 were sown in pots (10" x 10") filled with vermiculite and sand-mix in equal quantity. Eighteen pots in group of two (9 each) were sown with equal seeds of two cultivars. The pots were kept inside regulated chamber (optimum temperature regime 26/22°C, humidity 60%, photoperiod 16 h and light intensity 350 µmol m^{-2} s^{-1}) at National Phytotron Facility, IARI, New Delhi. Proper irrigation was provided at regular intervals. Both the cultivars in triplicate were exposed to heat stress (42°C for 2 h) at milky-ripe and mealy-ripe stages. A plant without treatment was used as control (22°C). Heat stress (HS) treatment was imposed as per the standard protocol[1]. Leaf samples were harvested at milky-ripe and mealy-ripe stages based on Feekes scale[15]. The collected samples (in triplicates) were immediately frozen in liquid nitrogen for subsequent analysis.

Data mining for identification of putative SS

The identification of novel heat-responsive genes in wheat at developing stage was done using *de novo* assembly in our laboratory. The raw data was assembled using Velvet and Oases v 2.0 and mined for the identification of putative transcripts showing homology with SS gene. We identified three putative SS transcripts in the assembled data.

Cloning of identified putative SS transcript
Designing of primers and RT-PCR

Transcripts predicted to be SS gene (identified by data mining) were used for the primer designing using the Genefisher2 designing software (http://bibiserv.techfak.uni-bielefeld.de/genefisher2/). The quality of the oligos was checked using Oligo Analyser (IDT, USA). Total RNA was isolated from immature endospermic tissues of HD2985 (thermotolerant) using RaFlex™ isolation kit (Genei, USA), following the instruction of the manufacturers. Precaution was taken to remove the starch granules, if any from the tissues before RNA isolation. The quality of isolated RNA was checked using Nanodrop (Thermo Fischer Scientific, USA).

The cDNA synthesis was carried out using the Revert Aid H minus cDNA synthesis kit (Fermentas, USA). The quality of the cDNA was checked on 1% agarose gel. The cDNA was used as template for the RT-PCR using the transcripts specific forward and reverse primers (Table 1). The amplicon of desired

size was gel eluted and used for the ligation in pGEMT$_{Easy}$ vector (Promega, USA) and transformation using *E. coli* DH5α competent cells. The cloned genes were sequenced using Sanger's dideoxy method.

In silico characterization of cloned transcripts

The sequenced genes were subjected to nucleotide based homology search using BLASTn tool of NCBI (http://blast.ncbi.nlm.nih.gov/). Similarly,BLASTp was also carried out to know the homology at protein level. The Expasy tool (http://www.expasy.org/) was used to find out the amino acid sequence, while Simple Modular Architecture Retrieval Tool (SMART) search (http://smart.embl-heidelberg.de/) was used to find out the conserved domain in the sequence.

ClustalW alignment (http://embnet.vital-it.ch/software/ClustalW.html) was performed using the nucleotide sequence reported from the plant and non-plant sources. Phylogeny tree analysis (http://www.phylogeny.fr/) was carried out in order to trace the evolution of the cloned genes. Other characterizations were performed using the bioinformatics tools available online.

Expression analysis of cloned transcripts

Total RNA was isolated from the control and HS-treated samples of both cultivars using RaFlex™ isolation kit (Genei, USA). cDNA synthesized using Revert Aid H minus cDNA synthesis kit was used as template (100 ngµl^{-1}) for the quantitative real-time PCR using the standard protocol[1]. In brief, reaction mix was prepared by adding 10 µl 2x SYBR mix, 0.4 µl 10µM forward and reverse primers each (Table 1), 100 ngµl^{-1}template and finally volume was make up to 20 µl by adding nuclease-free water. The PCR cycle was: 95°C for 3 min, 39 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 5 s, followed by plate read. The efficiency of primer binding was checked using dissociation curve analysis. The experiment was done using three biological and two technical replicates. The expression of β-actin gene was used as endogenous control for normalizing the data. Relative expression was calculated using Pfaffl method[16].

SS activity assay

0.5 g of sample were homogenized with a pestle in a pre-cooled mortar containing 5 ml frozen extraction medium [100 mmol L^{-1} HEPES-NaOH (4-2-hydroxyethyl-1-piperazine ethane sulfonic acid-sodium hydroxide)], pH 7.5, 2 mmol L^{-1} EDTA, 50 mmol L^{-1}

β-mercaptoethanol, 12.5% (v/v) glycerol). The supernatant obtained after centrifugation (10,000 *g*, 25 min, 4°C) was directly used for the activity assay of SS as per the standard protocol[1]. The activity was measured as the increase in absorbance at 340 nm after the addition of 1.4 IU hexokinase and 0.35 IU glucose-6-phosphate dehydrogenase. The standard curve of sodium pyruvate was used to calculate the activity of SS in nmole min^{-1} mg^{-1} protein.

Estimation of starch content in kernel tissue

Oven-dried mature endospermic tissue (100 mg) was extracted with 70% hot ethanol, centrifuged at 12000 rpm for 15 min and the residue was dried in oven. Residue was resuspended in 5.0 ml water and 7.5 ml of 52% perchloric acid, centrifuged at 12,000 rpm for 10 min and the supernatant was collected in fresh centrifuge tube. The sample was re-extracted using 5 ml perchloric acid and supernatant was pooled and volume was made to 100 ml with water. Suitable aliquot was used for glucose estimation using anthrone reagent. The intensity of the colour formed at 620 nm was used for glucose estimation using standard curve and value observed was multiplied by 0.9 in order to get the starch yield.

Guaiacol peroxidase (GPX) activity assay

GPX activity was determined following the method of Evers *et al.*[17] with slight modification. The oxidation of guaiacol into tetraguaiacol was estimated by measuring the absorbance at 470 nm against the reagent blank using extinction coefficient of 26.6 mM^{-1} cm^{-1}.

Activity assay of CAT

Fresh leaf material (1 g) was crushed in 6 ml of ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium-EDTA and 1% (w/v) polyvinylpyrrolidone (PVP). The homogenates were centrifuged at 10,000 *g* for 10 min. The tissue extracts were used for the quantification of soluble protein content and analysis of CAT. Coomassie blue dye-binding assay was used for protein estimation[18] and bovine serum albumin (BSA) for standard curve preparation. For assaying CAT activity, decomposition of H_2O_2 was followed by decline in the absorbance at 240 nm[19]. CAT activity was determined by following the consumption of H_2O_2 (extinction coefficient, 39.4 mM^{-1} cm^{-1}) at 240 nm over 3 min interval.

Estimation of free amino acid (FAA) content

The total FAA content was determined by ninhydrin test according to the method of Sircelj*etal.*[20]

A known weight of dry powdered plant material of each sample was extracted in 80% ethanol and centrifuged at 8,000 *g* for 15min. The supernatant containing the alcohol-soluble fraction was collected. A reaction mixture consisting 0.1 ml extract, 0.9 ml double-distilled water and 1ml ninhydrin reagent was shaken vigorously and then heated in a boiling water bath for about 20 min and added to 5 ml diluents (equal volumes of water and *n*-propanol). After cooling the tubes to room temperature, the colour intensity was measured at 570 nm. The blank set was prepared by adding 0.1ml of 80% ethanol in place of sample extract. The standard curve was prepared by using leucine as a standard amino acid. The results were expressed in terms of mg of amino acid/g of dry tissue.

Thermal characteristic of kernels under HS

The thermal characteristic of kernel tissues of both cultivars exposed to HS was correlated using total antioxidant capacity (TAC). The FRAP (ferric reducing antioxidant power assay) procedure described by Benzie and Strain[21] was followed for TAC assay. The values were expressed as concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mmol L^{-1} $FeSO_4$.

Statistical analysis

The experiment was conducted in a completely randomized design (CRD). Data were analysed using one-way analysis of variance (one-way ANOVA; p < 0.05). The standard errors were given in histograms.

Results and Discussion

Wheat is one of the important cereal grain providing 12% of carbohydrate and 40% of the calories in the diet. Terminal HS is one of the major problems in wheat growth, development and yield; 4% yield reduction has been reported for every 1°C rise in temperature. HS causes denaturation and aggregation of the enzymes, defragmentation of starch granules, small granules, shrinking of endosperm, empty pores inside kernel tissue, and distortion of seed compartments etc[1]. SSs are involved in the synthesis of amylose and amylopectin; being heat-labile, SS causes defragmentation of starch granule under HS[22].Although SS genes have been identified and exploited in the crops like rice, maize, sorghum etc., very limited information is available on SS genes from the wheat.

Identification and cloning of SS gene(s)

Data generated using next-generation sequencing (NGS) of control and HS-treated samples of HD2985

and HD2329 (NCBI BioProject Database: PRJNA171754) were mined for the identification of novel transcripts showing homology with SS genes. We identified 11 transcripts showing homology with SS genes reported from plant and non-plant sources. Transcript-specific primers were designed and RT-PCR was carried out using cDNA synthesized from total RNA of immature endosperm. We amplified, cloned and sequenced three transcripts of 768 (transcript_7326), 549 (transcript_24546) and 437 (transcript_38472) bp, respectively (Fig. S1). The gene sequences were submitted in National Center for Biotechnology Information (NCBI) GenBank with accession no. KM206143 (transcript_7326), KM206144 (transcript_24546) and KJ854903 (transcript_38472).

Five different SS isoforms — SSI, -II, -III, -IV and GBSS, each with a unique catalytic role in starch synthesis have been reported from different crops[23].Earlier, three distinct granule-bound isoforms of 60 kDa (the waxy gene product), 77 kDa and 100-105 kDa have been identified from wheat[24]. Also, *DU1* and *zSSI* genes predicted to be major SS genes have been reported from maize[22]. A putative GBSS gene involved in amylose synthesis has also been identified from banana fruit[25].

In silico characterization of cloned SS genes

The homology of cloned SS sequences using BLASTn (NCBI) showed that transcript_7326 had maximum homology with *GBSSII* (accession no. AF109395) reported from *T. aestivum*, followed by clone *NIASHv2070I19* (accession no. AK368223) cloned from sorghum. Transcript_24546 showed maximum homology with *SSIII* reported from *T. aestivum* (accession no. AF258608), followed by *Aegilopstauschii* (AF258609). Similarly, transcript_38472 showed maximum homology with *T. aestivum* cDNA clone *WT004_L10*(accession no. AK332724) reported from Chinese Spring cultivar, followed by *SSIIb* (accession no. EU333947) reported from *T. aestivum*.

The SSs reported from different plant sources retrieved from NCBI GenBank were subjected to ClustalW alignment along with the cloned transcripts individually, in order to find conserved regions as well as variations among the sequences. Twenty-six SS genes (accession nos. HG531811, HG531810, AB201445, AJ269503, BT009011, AF155217, AY133248, AB201447, AJ269502, U66377, XM_006656746, AY499410, AF019296, NM_001301653, EU620718,

EF090069, EF090074, XM_004964991, AK332724, KJ854903, EU333947, XM_003573051, XM_008682708, NM_001112544, NM_001111410 and XM_004953814) were used for the alignment along with transcript_38472 (Fig. 1a). We observed three prominent conserved regions — GGCAAGCGGCAGTGCAAGG, GCCCTGCAGCG and AGCT in the sequences. These regions are predicted to play important role in substrate binding (ADPG and glucan subunit) for enhancing the SS activity[26].

Similarly, SS sequences with accession nos. AF258608, FN179377, JN256950, JN256951, AF258609 were aligned along with transcript_24546. We observed three prominent conserved domains in the sequences — ATGGAAGGAA, TATATAAG and TATTCCCAAT which was different from domains reported in earlier section (Fig. 1b). Alignment of transcript_7326 did not show any significant results. Further, all the three cloned sequences were subjected to ClustalW alignment with AF109395, AK368223, AF486521, XM_003569190, FN179381, AY069940, NM_001112569, NM_001279972, EF221764, EF472248.

We found four prominent conserved domains — GGA, GG, G and A in the sequences (Fig. 2).The alignment results obtained with the cloned SS genes were used for the generation of phylogeny tree. The SS identified were grouped in four broad families. Based on the sequence homology, transcript_7326 was grouped under family-III along with GBSS and other SS reported from wheat and sorghum (Fig. S2). Similarly, transcript_38472 were broadly classified into four distinct groupsandwere grouped under family-II, showing homology with SSIIb from *T. aestivum* (Fig. 3).

Domain search using the Simple Modular Architecture Retrieval Tool (SMART) showed the presence of Glycosyl_transferase_1, Glycosyl_transferase_1_4 and Glycosyl_transferase_5 domains in the respective transcripts.The length and homology of all three putative SS transcripts with glycosyltransferase domains is characteristics of SS enzymes that use ADP-glucose as substrate[23]. These catalytic domains play very important role in sugar moiety transfer in order to synthesize short glucan chain called amylose, forming major portion of endosperm starch[27].

Expression profiling of cloned SS genes under HS

The transcripts identified in present study (transcript_7326, transcript_24546 and transcript_38472) predicted to be putative SS were characterized for their expression during grain-filling (milky-ripe and mealy-ripe) stages under HS. The relative expression of transcript_7326 during milky-ripe stage was 0.36 (HD2985) and 0.20-fold (HD2329) and during mealy-ripe stage, 0.95 (HD2985) and 0.70-fold (HD2329) in HS-treated samples, compared to control (Fig. 4). Though down-regulation of transcript_7326 was observed under HS in both the cultivars during milky-ripe and mealy-ripe stages, higher expression was observed in mealy-ripe, compared to milky-ripe under HS. The down-regulation of transcript_7326 was observed less in thermotolerant cultivar, compared to thermosusceptible under HS. Similarly, transcript_24546 showed 0.9 (HD2985) and 0.38-fold expression during milky-ripe and 1.1 (HD2985) and 0.7 (HD2329) fold during mealy-ripe in HS-treated samples compared to control. The up-regulation of transcript_24546 in HD2985 and down-regulation in HD2329 were observed under HS; expression was higher during mealy-ripe, compared to milky-ripe stages under HS.

Similar expression pattern was observed in case of transcript_38472 during milky-ripe and mealy-ripe stages under HS (Fig. 4). The transcripts predicted to be SS showed down-regulation during grain-filling stage, except transcript_24546 which showed up-regulation in thermotolerant cultivar under HS. The relative expressions of cloned SS genes were observed significant in response to HS-treatments. Earlier, Hirose and Terao[28] have identified ten SS genes from the rice and based on their expression divided them into three groups: (i) early expressers (*SSII-2*, *III-1*, *GBSSII*), (ii) late expressers (*SSII-3*, *III-2*, *GBSSI*), and (iii) steady expressers (*SSI*, *II-1*, *IV-1*, *IV-2*).

Effect of HS on SS activity

Samples (control and HS-treated) from both the cultivars at milky-ripe and mealy-ripe stages were used for the SS activity assay. A decrease in the SS activity was noticed in both the cultivars under HS during milky and mealy-ripe stages (Fig. 5a). During milky-ripe stage, higher SS activity was observed in HD2329, compared to HD2985 under control condition, whereas HS caused abrupt decrease in the activity of SS in HD2985; percentage decrease was less in HD2329, compared to HD2985. During mealy-ripe stage, a decrease in SS activity was observed under HS in both the cultivars; percentage decrease was higher in HD2329 compared to HD2985. The variation in the SS activity in both the cultivars was

significant at grain-filling stage. Prolonged exposure of elevated temperature causes loss of various enzymes activity. The increase in the temperature is reported to adversely affect the SS activity required for the synthesis of amylopectin in wheat endosperm. SS is the key regulatory enzyme of starch biosynthesis in developing wheat grain[29].

Earlier, it is reported that elevation in temperature during grain-filling stage is an important yield limiting factor in wheat[29]. Scanning electron microscopy (SEM) of starch granules has also revealed that HS disrupts the synthesis and filling of starch granules inside the endosperm[1].

Effect of HS on starch accumulation

For starch estimation, samples (control and HS) from both cultivars at milky-ripe, mealy-ripe and kernel hardening stages were used (Fig. 5b). During milky-ripe, significant decrease in starch accumulation was observed under HS in both the cultivars; among control, starch accumulation was higher in HD2985, compared to HD2329. Similarly, percent increase in the starch content was higher at mealy-ripe stage in both the cultivars; HD2985 showed more accumulation, compared to HD2329. Both the cultivars exhibited decrease in the starch content during mealy-ripe under HS (Fig. 5b). In case of HD2329, however, the decrease was non-significant.

During kernel hardening stage, maximum starch accumulation was observed in both the cultivars under control condition, compared to earlier stages. Overall, HD2985 showed maximum starch accumulation both under control and HS conditions. HS affects the rate of assimilate transport and grain-filling duration affecting the endosperm development[30,31]. It is also reported to cause significant decline in starch content and marked build-up of total free sugars and protein content in grains of different wheat cultivars[32].

Effect of HS on GPX activity

GPX activity was assayed in both cultivars exposed to HS during milky-ripe and mealy-ripe stages. Significant increase in GPX activity was observed during milky-ripe in HD2985 under HS; percentage increase was more in thermotolerant, as compared to thermosusceptible (Fig. 6a). Similarly, HS decreased GPX activity in response to HS during mealy-ripe, whereas HD2329 showed increase in GPX activity under HS. Thus, GPX activity in the thermotolerant cultivar was more sensitive to HS, whereas in

thermosusceptible cultivar, it was resistant to elevated temperature (Fig. 6a).

A possible mechanism that may be involved in developing tolerance against abiotic stress is increase in activity of the antioxidant pathways[1]. It is reported that cell membrane damage due to HS can be recovered by the association of the GPX activity[33]. The present study showed that GPX activity was significantly increased by high temperature, which was consistent with the studies reporting the increased GPX activity in response to different types of abiotic stresses[34-36].

Effect of HS on CAT activity during grain-filling

CAT activity was assayed in fresh leaf, stem and endosperm from both cultivars under control and HS conditions (Fig. 6b). The maximum activity was observed in HS-treated leaf of HD2329, compared to control. Similarly, in case of stem, maximum CAT activity was observed in HS-treated sample of HD2985, compared to HD2329. During milky-ripe, non-significant differences in CAT activity in leaf, stem and endosperm tissues was observed in control samples of both cultivars. Maximum CAT activity was observed in control and HS-treated endospermic tissues of HD2985. In case of HD2329, decrease in CAT activity was noticed in control and HS-treated samples during milky-ripe stage (Fig. 6b).

Earlier study has observed that CAT activity shows an initial increase with increase in the temperature and after certain limit (50°C) its activity declines. The activity of CAT has been used as marker for screening thermotolerance level of wheat germplasm, as thermotolerant cultivar shows better tolerance to HS, as compared to thermosusceptible[1].

Effect of HS on TAC during grain filling

Thermotolerance of leaf, stem and endosperm was analysed during milky-ripe and mealy-ripe stages by estimating the TAC of both the cultivars under differential HS (Fig. 7). In leaf tissues during milky-ripe, significant increase in TAC was observed under HS, compared to controls in both the cultivars. The maximum TAC was observed in leaves of HS-treated HD2985, compared to HD2329. Similarly, TAC in stem of both the cultivars during milky ripe was very low, compared to leaf; variations were significant. Similar pattern of TAC was observed in endospermic tissue of both the cultivars during milky-ripe stage. HS-treated endospermic tissues of HD2985 showed maximum TAC, compared to other treatments (Fig. 7).

The plants exposed to HS have maximum TAC at 35-40°C in tolerant varieties and at 30°C in susceptible one[37]. TAC differs depending upon tolerance or susceptibility of different crop varieties, their growth stages and growing season[38,39].

During mealy-ripe stage, maximum TAC was observed in HS-treated leaf of HD2985 compared to other treatments. Significant TAC was observed in case of leaves of HD2985 (control and HS) and HD2329 (control), whereas HS-treated HD2329 showed abrupt decrease in TAC (Fig. 7). Similarly, TAC in stem and endospermic tissues of both cultivars during mealy-ripe showed very low TAC, compared to leaf tissue. In case of stem and endosperm, the maximum TAC was observed in HS-treated HD2985; significant variations were observed in stem and endosperm tissues of both cultivars under HS. It is reported that TAC under stress condition prevents damage and plays significant role in the tolerance of plants[36,40].

Effect of HS on FAA during grain-filling

Samples (leaf, stem and endospermic tissues) from control and HS-treated HD2985 and HD2329 were analysed for the accumulation of FAA during milky-ripe and mealy-ripe stage (Fig. 8). During milky-ripe stage, significant increase was observed in the FAA accumulation in leaf and stem under HS in both the cultivars. The percentage increase in the leaf FAA under HS was observed higher in HD2985, compared to HD2329. Earlier a linear increase in the FAA has been reported in *Brassica juncea* with the induction of drought stress[41], which was in agreement with present findings.

Similarly, increase in the FAA content was observed in stem tissues of both the cultivars at milky-ripe stage under HS. Maximum FAA was observed in endospermic tissues of HD2985 in response to HS during milky-ripe stage, compared to HD2329; variations observed were highly significant. Also, FAA estimation in samples (control and HS) during mealy-ripe showed maximum accumulation in leaf and endospermic tissues of HD2985, compared to HD2329 (Fig. 8). In thermosusceptible cultivar, maximum FAA was observed in endospermic tissues and minimum in stem under HS. In cowpea, total FAA levels are reported to increase approx. 1.5-fold after 24 h at 42°C; heat shock leads to release of amino acids into the medium, indicating that damage of the integrity of the plasma membrane[42].

Correlation between protein and FAA in endospermic tissue under HS

The endospermic tissue from control and HS-treated HD2985 and HD2329 was used for the estimation of protein and FAA during mealy-ripe stage. Significant increase in protein content, as well as FAA was observed in both the cultivars under HS (Fig. S3). The findings were in agreement with the report of Asthir and Bhatia[32]. The percentage increase in FAA under HS was highly significant, compared to protein in both the cultivars. The FAA accumulation in HD2329 was less, compared to HD2985 in both the control and HS-treated endospermic tissues. Protein biosynthesis is reported to increase under HS[43]. The increase in accumulation of FAA and protein under HS might be due to the disruption of starch biosynthesis, which favors nitrogen metabolism leading to higher amino acid and protein accumulation[44]. Proline constitutes 80% of the FAA and acts as an osmolyte, stabilizing the sub-cellular structure, scavenging free radicals and maintaining redox potential under stress[45].

Conclusion

Heat stress is one of the major detrimental factors reducing the yield and quality of wheat. Grain-filling is considered as most sensitive stage for HS, as it alters the activity of the enzymes involved in starch biosynthesis pathway and causes shrivelled grains. Here, we report cloning of three putative SS genes (transcript_7326, transcript_24546 & transcript_38472) from HD2985 cultivar of wheat. Expression analysis showed down-regulation of SS genes in response to HS in both HD2985 and HD2329 during grain-filling stage; transcript_24546 showed up-regulation in HD2985. SS activity assay showed decrease in activity under HS during milky-ripe and mealy-ripe stages. Starch content was found to be higher in HD2985 during mealy-ripe stage, compared to HD2329. Similarly, significant variations were observed in GPX and CAT activities and overall TAC in samples at milky and mealy ripe stages; HD2985 had better tolerance level compared to HD2329. To conclude, heat-labile SS is protected by series of antioxidant defence system which maintains its activity under HS by modulating the tolerance level of leaves and endospermic tissues harbouring SS enzymes.

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Table 1—Primers used for the cloning and expression profiling of identified
putative SS genes (transcripts_7326, transcript_24546 and transcript_38472)*
using *de novo* assembly

Gene ID	Primer sequences (5'-3')	T_m (°C)
Tra_7326(F)	GTGTTGAGTTGGATGGCGTCCTTCG	60.4
Tra_7326(R)	CTCGACTCCTAGCCCAAGAAGAGCCT	63.7
qTra_7326(F)	TGCAAGCTGAAGTTGGATTG	59.9
qTra_7326(R)	CCGGTATGGCTGCAATTAGT	59.9
Tra_24546(F)	CAGAGAGCCTAACATGCACATTAG	56.6
Tra_24546(R)	CTCAACAGAGCAATCGGCGCTT	60.3
qTra_24546(F)	ATGCGCTTAACCATTTCCAG	60
qTra_24546(R)	AGGGGGAACAAAGCAGAAAT	60
Tra_38472(F)	GCCCTGTAGATGACTTCAACATC	60
Tra_38472(R)	CCGATGAGCGGCACGTCAT	57.5
qTra_38472(F)	GAGCTGAAGACGATGGAAGG	59.9
qTra_38472(R)	AGCGTGTCTCGGGTGTAGTT	59.7
β-Act(F)	GCGGTCGAACAACTGGTATT	52.2
β-Act(R)	GGTCCAAACGAAGGATAGCA	51.7

*Tra, Transcript, q, primer used for the real-time PCR, F, forward primer, R, reverse primer, Act,β-actin; expression profiling was carried out using CFX96 platform (Bio Rad, USA).

Fig. 1—Multiple ClustalW alignment of transcript_38472 (a)and transcript_24546 (b) cloned in this study along with other SS genes reported from plant sources [SS sequences were retrieved from the GenBank and alignment was carried out using ClustalW2 alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Three prominent conserved regions were observed and predicted to play role in substrate binding (Fig. 1a). Three prominent conserved regions were observed (Fig. 1b)]

Fig. 2—Multiple ClustalW alignment of transcript_7326, transcript_24546 and transcript_38472 cloned in present study along with other SS genes reported from plant sources [Sequences were retrieved from the GenBank and alignment was carried out using ClustalW2 alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/).Eight prominent conserved regions were observed in the sequence]

Fig. 3—Phylogeny analysis of cloned putative SS gene (transcript_38472) from HD2985 (thermotolerant) cultivar under HS [Phylogeny tree was drawn using Phylogeny. Fr tool (http://www.phylogeny.fr/).Based on phylogeny analysis, SSs were classified into four families with transcript_38472 grouped under family II]

Fig.4—Differential expression profiling of identified putative SS genes (transcripts_7326, transcript_24546 and transcript_38472) in thermotolerant (HD2985) and thermosusceptible (HD2329) cultivars at grain-filling stage under HS [Plants were exposed to HS (42°C, 2 h)at milky-ripe and mealy-ripe stages. Expression of actin gene was used as endogenous control. Relative expression was calculated using Pfaffl method. Vertical bars indicate *S.E* (n=3)]

3

Fig. 5—Effect of HS on SS activity and starch accumulation in HD2985 (thermotolerant) and HD2329 (thermosusceptible) cultivars at grain-filling stage [(A) SS activity assay at milky-ripe and mealy-ripe stages, and (B) starch accumulation at milky-ripe, mealy-ripe and kernel hardening stages. C, control (22°C), HS, heat stress (42°C, 2 h).Plants were exposed to HS at different sub-stages of grain-filling; stages were selected based on the Feekes scale. Samples were collected in triplicates. Different letters above each bar indicate significant difference ($p < 0.05$) between treatments (one-way ANOVA).Vertical bars indicate S.E (n=3)]

Fig.6—Tissues-specific activity assay of GPX (a)and CAT (b) in thermotolerant (HD2985) and thermosusceptible (HD2329) cultivars at grain-filling stage under HS [(A) Milky-ripe stage, and (B) mealy-ripe stage. Leaf, stem and endospermic tissues were used for activity assay. Control (C), 22°C, HS, heat stress (42°C, 2 h). Different letters above each bar indicate significant difference ($p < 0.05$) between treatments (one-way ANOVA).Vertical bars indicate S.E. (n = 3)]

Fig. 7—Estimation of total antioxidant capacity (TAC) in HD2985 (thermotolerant) and HD2329 (thermosusceptible) cultivars exposed to HS during grain-filling stage [(A) Milky-ripe, and (B) mealy-ripe stages. Control (C), 22°C, HS, heat stress (42°C, 2h). Different letters above each bar indicate significant difference ($p < 0.05$) between treatments (one-way ANOVA).Vertical bars indicate S.E. (n = 3)]

Fig.8—Changes in free amino acid (FAA) accumulation in HD2985 (thermotolerant) and HD2329 (thermosusceptible) cultivars exposed to HS during grain-filling stage [(A) Milky-ripe, and (B) mealy-ripe stages. Control (C), 22°C, HS, heat stress (42°C, 2 h). Different letters above each bar indicate significant difference ($p < 0.05$) between treatments (one-way ANOVA).Vertical bars indicate S.E. (n=3)]

Supplementary Material

Fig. S1—Nucleotide sequence of cloned transcripts predicted to be SS and identified from the *de novo* transcriptome sequencing data of HD2985 cultivar [Sanger's di-deoxy method was followed for the sequencing. Sequence was submitted in NCBI GenBank with accession nos. KM206143 (transcript_7326), KM206144 (transcript_24546) and KJ854903 (transcript_38472)]

Fig. S2—Phylogeny analysis of cloned putative SS gene (transcript_7326) from HD2985 (thermotolerant) cultivar under HS [Phylogeny tree was drawn using Phylogeny. Fr tool (http://www.phylogeny.fr/).Based on phylogeny analysis, SSs were classified into four families with transcript_7326 grouped under family IV]

Fig. S3—Correlation of protein and free amino acid (FAA) accumulation in endospermic tissue of HD2985 and HD2329 exposed to HS during grain-filling stage [Control (C), 22°C, HS, heat stress (42°C, 2 h).Different letters above each bar indicate significant difference ($p < 0.05$) between treatments (one-way ANOVA).Vertical bars indicate S.E. (n = 3)]