

Original Research Article

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Wheat Oxygen Evolving Enhancer Protein: Identification and Characterization of Mn-Binding Metalloprotein of Photosynthetic Pathway Involved in Regulating Photosystem II Integrity and Network of Antioxidant Enzymes under Heat Stress

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ABSTRACT

PsbO is a ubiquitous extrinsic protein of photosystem II required for providing proper environment for water splitting activity of photosystem II. Exposure of wheat to heat stress initially reduces the rate of photosynthesis by disintegrating the photosystem II, primarily by disruption of the oxygen evolving complex (OEC). Here, we identified and cloned putative PsbO gene of 1 kb from wheat cv. HD2329 using de novo assembly. Expression analysis showed higher expression of *PsbO* transcript in HD2985, as compared to HD2329 during pollination and grain-filling stages. We observed significant variations in the expression of *PsbO* in response to differential HS. A positive correlation was established between the expression of *PsbO* and activity of antioxidant enzymes like superoxide dismutase, catalase and guaiacol peroxidase in response to heat stress in contrasting wheat cvs. at different stages of growth. Photosynthetic rate was observed slightly higher in thermotolerant cultivar at different stages, as compared to susceptible. There is, however, need to discover many more isoforms of *PsbO* from wheat in order to characterize their role in the modulation of carbon assimilatory pathway under heat stress. Maintaining the integrity of PSII under HS using PsbO will pave the way for the development of 'climate-smart' wheat.

Keywords

Catalase, Heat stress, Peroxidase, photosynthetic rate, IRGA, PsbO, qRT-PCR, Superoxide dismutase, Total antioxidant activity

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Introduction

Photosynthesis is the most sensitive physiological process at elevated temperature (Wahid *et al.*, 2007). Exposure of cereals to long term high temperature results in a

reduction in photochemical efficiency of Photosystem II (PS II) due to photo inhibition and damage to chloroplast (Hasanuzzaman *et al.*, 2013). Heat stress drastically reduces the rate of photosynthesis by disintegrating the photosystem II (Sharkey, 2005). Photosystem

II complex is a heat-labile pigment-protein complex which is involved in the electron transport of the photosynthetic process and responsible for the light-induced water oxidation to produce molecular oxygen. The heat induced inactivation of this complex is caused principally by dissociation of the oxygen evolving complex (OEC) (Kimura *et al.*, 2002). The activity of the Oxygen Evolving Complex (OEC) of PS II is often lost before the rest of the reaction centre loses its activity (Aro *et al.*, 1993). The largest extrinsic subunit protein of photosystem II (PSII) is PsbO. It is the second abundant hydrophilic protein on the world, which is next to the most abundant RuBisCO subunit protein (Henmi *et al.*, 2004). Evidence suggests that PsbO protein have important role in regulation of PSII activity. Removal of PsbO leads to destabilise Mn-cluster and Mn ions are released (Enami *et al.*, 2000), for that reason this protein is also called 'Mn stabilising protein' Bricker, (1992) reported that PsbO protein functions to control the chloride and calcium requirements for oxygen evolution. The oxygen evolution requirements for both of these ions are significantly lowered in the presence of the PsbO component. Evidence has been presented that the PsbO protein binds GTP with high affinity and functions as a GTPase (Lundin *et al.*, 2007). In this role, it has been predicted in controlling the phosphorylation state of the D1 protein (Lundin *et al.*, 2007a). Since the phosphorylation level of the D1 component appears to be coupled with its efficient PS II turnover, it was proposed that the PsbO protein may be an important regulatory component of this process (Lundin *et al.*, 2008). McConnell *et al.*, (2007) reported that the association of carbonic anhydrase activity with PS II is highly variable. Carbonic anhydrase activity associated with PS II membranes has lost upon purification of an oxygen-evolving PS II core complex which contained the PsbO component. Kim *et al.*,

(2015), it was found that PsbO had higher H₂O₂ scavenging activity, which proved to be the result of enzymatic antioxidants rather than non-enzymatic antioxidants. It was found that PsbO act as a structural template and a sensor of the hydrogen-bonding network which is required for proper water oxidation of photosystem (Offenbacher *et al.*, 2013). Recently, Takahashi *et al.*, (2017) reported that PsbO is the only protein nitrated when *Arabidopsis thaliana* thylakoid membranes are incubated in a buffer bubbled with nitrogen dioxide (NO₂) that leads to the inactivation of photosystem II. The mutation in aspartic acid (D157) in the *PsbO* gene encoding the photosystem II Manganese-stabilizing protein from spinach, exhibit near-wild-type PSII binding mechanisms, but are significantly impaired in O₂ evolution activity and Cl⁻ retention by PSII (Popelkova *et al.*, 2009).

The growing threat of climate change is already having a considerable influence on agricultural production worldwide as heat stress cause significant yield losses with great challenges for future food security (Christensen and Christensen, 2007). Wheat (*Triticum aestivum* L.) is a staple food crop which is very sensitive to high temperature (Slafer and Satorre 1999). It was reported that in some wheat varieties there is a loss of 10-15% of yield with an increase of every 5°C in temperature (Burrell 2003). Quality and quantity of wheat grains is also reduced by heat stress (Viswanathan and Khanna-chopra 2001). Photosynthesis is harmfully affected by heat stress in a number of ways (Kumar *et al.*, 2016). In that Oxidative stress is a major outcome that leads to the formation of hydroxyl radicals (OH[•]), superoxides (O₂⁻) and hydrogen peroxide (H₂O₂) which are commonly called reactive oxygen species. Hence, their detoxification by antioxidant systems is important for protecting plants against heat stress (Asada, 2006; Suzuki and Mittler, 2006). Otherwise the ROS may

involved in destroying D1 protein of photosystem II (Yamamoto *et al.*, 2008), and inhibits the repairing of photodamaged PSII (Murata *et al.*, 2007), that leads to inactivating of PSII (Yamashita *et al.*, 2008). This stress associated ROS toxicity considered to be one of the key reason of decreased crop productivity (Vadez *et al.*, 2012).

In present investigation, we have identified, cloned and characterized a novel *PsbO* gene from wheat *cv.* HD2329 and correlation was established between the expression of *PsbO* and different biochemical and physiological parameters under HS.

Materials and Methods

Plant material and heat stress treatment

Two wheat cultivars *i.e.* HD2985 and HD2329 were used in this study. Seeds were sown in 18 pots (nine for each variety) having equal quantity of perlite to FYM mixture inside the regulated chamber (temperature $22\pm 3^{\circ}\text{C}$; relative humidity of 75% and 8 hours) in the National Phytotron Facility, IARI, New Delhi. Irrigation was done at regular intervals, and plants at the pollination and grain-filling stage were selected (based on the Feekes scale; three pots from each variety) were exposed to heat stress of 30°C and 38°C for 1 h, whereas other three pots in each group served as control ($22\pm 3^{\circ}\text{C}$). The heat stress was given in a sinusoidal mode using microprocessor-regulated controller with an increase of $1^{\circ}\text{C}/10$ minutes till the temperature reaches the 30° or 38°C , and it was maintained for 1 hours.. Samples were collected in triplicates from both the cultivars frozen in liquid nitrogen for further downstream analysis.

Isolation and cloning of *PsbO* from Wheat

An RNA-Seq experiment was executed in our lab for the identification of novel heat-

responsive stress-associated genes in wheat *cvs.* HD2985 and HD2329 at developing stage using *de novo* transcriptome sequencing on Illumina HiSeq 2000 platform. The raw data generated using Next-Generation Sequencing (NGS) of control and HS treated samples of HD2985 and HD2329 cultivars of wheat (NCBI BioProject Database: PRJNA171754) was assembled using Velvet and Oases v 2.0 and was mined for the identification of *PsbO* transcripts based on the presence of conserved domain in the transcripts as well as homology based search. Transcript predicted to be *PSBO* gene (identified by data mining) were used for designing primers. Transcript specific primers were designed using Genefisher2 primer designing software, and the quality of the oligos was checked using Oligo Analyzer (Integrated DNA Technologies, USA) (Table 1). Total RNA was extracted from the leaves of wheat *cv.* HD2329 using Trizol reagent (Invitrogen, UK). Quality of isolated RNA was checked using Nanodrop (Thermo Fischer Scientific, USA). cDNA synthesis was carried out by using Revert Aid H Minus First stand cDNA synthesis kit (Fermentas, UK) as per the instruction given by the manufacturer's. The quality of cDNA was checked on 1% agarose gel. RT-PCR amplification was carried out using the cDNA template by using transcript specific primers. The amplified product was purified using the PCR cleanup kit (Promega, USA), and ligated into pGEM-T_{Easy} vector using ligase enzyme (Promega, USA) and transformation using *E. coli* DH5 α competent cells. The cloned genes were sequenced using Sanger's di-deoxy method.

***In silico* analysis of *PsbO* gene**

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 and further Simple Modular Architecture Retrieval Tool (SMART) search (<http://smart.embl-heidelberg.de/>) was used to find out the conserved domain in the sequence. Rampage was used to confirm predicted protein structure. iPSORT was used for sub cellular localization. Ensemble Plant tools were used for chromosomal localization of gene. ProtParam was used for computation of several physical and chemical parameters. ORFfinder NCBI was used for identification of open reading frame of cloned gene. Protein 3D modeling was done using PHYRE2 Protein Fold Recognition Server.

RNA extraction and quantitative real time PCR (qRT-PCR)

Total RNA was isolated using Trizol method from both cultivars from control and HS-treated samples. CDNA synthesis was carried out by using Revert Aid H Minus First stand cDNA synthesis kit (Fermentas, UK), and quality and quantity was ensured by using Nanodrop. Quantitative RT-PCR was carried out by using KAPA SYBR Green qPCR master mix on a Bio-Rad CFX96 machine. Wheat β -Actin gene (accession no.AF282624) was used as the internal control gene. Relative expression levels of *PsbO* gene was quantified by using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

Measurement of photosynthetic rate

Wheat *cvs.* HD2329 and HD2985 were used for the IRGA analysis under control ($22\pm 3^\circ\text{C}$) and HS (30°C and 38°C , 2h) during the pollination and grain-filling stage (Feekes - 11.1). The plants were subjected to infra-red gas analyzer (IRGA) for analyzing the effect of HS on the photosynthesis (LiCor 6400, LiCor Inc., USA), and the observations were recorded following Long and Bernacchi, (2003). Fully expanded flag leaf was used for the IRGA analysis, and the readings were

taken in triplicates between 11 am to 12 pm. Photosynthesis was measured at constant saturating light of $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$. Measurements of leaf R dark were made on dark-adapted leaves after 30 min of dark adaptation to achieve steady- state R dark. The calculation was carried out as per suggested protocol by Kumar *et al.*, (2017).

Estimation of antioxidant enzymes

Guaiacol peroxidase (GPX) activity assay

Samples (control and heat stressed sample) collected from wheat *cvs.* HD2329 and HD2985 were used for the guaiacol-peroxidase (GPX) activity assay following the method of Evers *et al.*, (1994) with slight modification. Fresh leaf material (1 g) was crushed in 5 mL of ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium-EDTA and 1% (w/v) polyvinyl-pyrrolidone (PVP). The homogenates were centrifuged at 10,000 g (4°C) for 10 min. The tissue extracts were used for the quantification of soluble protein content and analysis of peroxidase activity. Coomassie blue dye-binding assay was used for protein estimation and bovine serum albumin (BSA) for standard curve preparation. The oxidation of guaiacol into tetraguaiacol was estimated by measuring the absorbance at 470 nm against the reagent blank, using extinction coefficient of $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

Estimation of superoxide dismutase activity

The samples (Control and heat stressed sample) collected from wheat *cvs.* HD2329 and HD2985 were used for the SOD activity assay based on the Nitrobluetetrazolium (NBT) method. The assay mixture contained 1.2ml of sodium pyrophosphate buffer, 0.1 ml of PMS, 0.3ml of NBT, 0.2ml of the enzyme preparation and water in a total volume of 2.8 ml. The reaction was initiated by the addition of 0.2 ml of NADH. The mixture was

incubated at 30°C for 90 seconds and arrested by the addition of 1.0ml of glacial acetic acid. The reaction mixture was then shaken with 4.0ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560nm in a spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute.

Catalase activity assay

Activities of catalase enzyme were measured as described by Chance and Maehly, (1955). Fresh leaf material (1 g) was crushed in 5 mL of ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM sodium-EDTA and 1% (w/v) polyvinyl-pyrrolidone (PVP). The homogenates were centrifuged at 10,000 g (4°C) for 10 min. The tissue extracts were used for the quantification of soluble protein content by using Bradford method and analysis of catalase activity. Catalase activity was measured in a reaction mixture (3 mL) containing 100 mM Na₂HPO₄ buffer pH 6.8 (2 mL), 30 mM H₂O₂ (0.5 mL) and 0.5 mL enzyme. For assaying CAT activity, the decomposition of H₂O₂ was followed by decline in the absorbance at 240 nm as catalase enzyme catalyzes the reaction.

Estimation of total antioxidant capacity

The FRAP (Ferric reducing antioxidant power assay) procedure for estimating total antioxidant activity was followed as described by Benzie and Strain (1999) with slight modification. Aliquots of 100 µL sample were mixed with 3 mL FRAP reagent and the absorbance of the reaction mixture at 593 nm was measured spectro photometrically after incubation at 37°C for 10 min. For the calibration curve, five different concentration of FeSO₄·7H₂O (100, 750, 500, 250 and 125 µmol/L) were used and the absorbance were

measured at 593 nm. The values were expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mmol/L FeSO₄.

Results and Discussion

Molecular cloning and *in-silico* characterization of candidate *PsbO* gene

Whole transcriptome sequencing of wheat was carried out in our lab for the identification of novel heat-responsive genes at grain-filling stage using *de novo* assembly. This NGS data was mined for the identification of novel *PsbO* transcripts based on the domain search and homology with *PsbO* gene. The identified *PsbO* transcripts mined from the NGS data were characterized for their Digital Gene Expression (DGE). Based on the DGE, transcript (CDS_52998_unigene_92869) which showing maximum DGE, targeted for the cloning. Transcript-specific forward and reverse primers were used along with cDNA template synthesized from the HS-treated HD2329 for the RT-PCR. The amplicon was excised from the gel, purified and the purified amplicon was ligated in pGEM-T Easy vector and transformed in *E. coli* DH5a strain. Positive colonies were selected based on the blue white colony selection. The isolated plasmids were subjected to restriction analysis (RE: *EcoRI*) in order to check the release of the target *PsbO* gene. We observed release of ~1.0 kb fragment from the plasmid on 0.8% agarose gel. The isolated plasmid showing release of target gene was sent for the sequencing by Sanger's di-deoxy method. Sequencing followed by curing showed the presence of 987 nucleotides in the amplified sequence. BLASTn (NCBI) search showed maximum homology with *Triticum aestivum* cDNA clone WT007 L07 cultivar: Chinese spring (acc. no. AK331517.1) followed by *Aegilopstauschii subsp. Tauschii* Oxygen evolving enhancer protein 1, chloroplastic

(LOC109772509) mRNA, (acc. no. XM020331189.1). BLASTp analysis showed maximum (100%) homology with Oxygen evolving enhancer protein 1 reported from *Aegilopstauschii subsp. Tauschii* (acc. no. XP 02018677778.1) followed by 99% with Predicted protein reported from *Hordeumvulgare subsp. vulgare* (acc. no. BAJ89638.1). Gene sequence was submitted in National Centre for Biotechnology Information (NCBI) GenBank with acc. no. KY014425.

Open reading frame was predicted using ORF finder (NCBI), A total of 6 ORF were predicted, longest ORF was of 987 base pairs (bp) spanning from 1 to 987 bp which encodes for 328 amino acids. Conserved domain search showed the presence of MSP (Manganese stabilizing protein) superfamily (accession no. PLN00037) with a bit score of 486.40 and Conserved Domain length of 232 amino acids (Fig. 1a). The cloned *PsbO* gene sequence was mapped on to the genome of *Triticum aestivum* available in Ensembl plants (http://plants.ensembl.org/Triticum_aestivum), and was observed to be localized on the chromosome 2B. Further, the amino acid sequence of the cloned gene was obtained through Expasy translate tool, and the sequence used for the prediction of the protein model using Protein Homology Analogy Recognition engine 2.0 (Phyre 2.0). The PsbO protein showed the presence of 14% α -helices, 42% β -strand and 56% disorder structure, which validates the report of (Svensson *et al.*, 2002; Popelkova *et al.*, 2009). Protein was modelled based on template model of cryo-em structure of spinach photosystem2-light harvesting complex 2(psii-lhcii) (Fig. 1b). 22 serine residues, 12 tyrosine and 3 threonine were predicted to be phosphorylation site in the putative protein using NetPhos 3.1 server (<http://www.cbs.dtu.dk/services/NetPhos/>). these predicted sites in this cloned gene

confirms the earlier report by Yang *et al.*, (2003) and Lundin *et al.*, (2007) who reported that PsbO protein is regulated by phosphorylation. (Fig. 1c). In Physical and chemical properties of protein, theoretical pI was found to be 5.75 and aliphatic index 70.58, instability index 37.05 was retrieved through Expasy ProtParam tool. Chloroplastic transit peptide sequence of 21 amino acids was observed on protein which shows their cellular localization. Existence of the predicted protein structure was confirmed by using rampage in which 98% of amino acid residue comes under the favored region of Ramchandran plot (Fig. 1d).

Expression profiling of *PsbO* gene in wheat under heat stress

The cloned *PsbO* gene was characterized for their expression during pollination and grain filling stage under differential heat stress in thermotolerant (HD2985) and thermosusceptible (HD2329) *cv.* of wheat using quantitative Real-Time PCR (qRT-PCR). Total RNA was isolated from freshly harvested leaf samples and purity was checked on 1.2% denaturing agarose gel. In pollination stage relative expression of putative *PsbO* transcripts was observed at 30°C for 1h in both the *cv.*; percent increase was observed significantly higher in thermotolerant cultivar, HD2985, as compared to thermosusceptible, HD2329. In pollination stage wheat cultivar HD2985 showed relative expression of 1.74 fold in response to HS of 30°C for 1 h, as compared to control. Similarly, thermosusceptible *cv.* HD2329 showed 1.54-fold increase in the expression. Similarly, we observed 1.90 fold increase in the expression and 1.59 fold in HD2329 HD2985 in response to 38°C, 2 h during pollination stage. Similar pattern was observed during grain-filling stage. HD2985 showed relative expression of 2.0 fold in response to HS of 30°C for 1h, as compared to control (Fig. 2). HD2329 showed

1.93-fold increase in the expression under HS of 30°C for 1h. HD2985 during grain-filling showed expression of 2-fold in response to HS of 38°C for 1 h. Similarly, HD2329 showed 1.75-fold increase in the expression in response to HS of 38°C for 1 h. Pawlowicz *et al.*, (2012) observed that increase in the accumulation of *PsbO* gene in Tall fescue (*Festuca arundinacea*) in response to drought stress. A significant, positive, and linear correlation has been reported by Liu *et al.*, (2015) in which they have showed the increase in digital fold expression of *PsbO* gene under heat and drought stress in wheat. Choulet *et al.*, (2014) also reported the increased digital expression of *PsbO* gene in different tissues of wheat.

Alterations in the photosynthesis-associated parameters in wheat under HS

We observed significant decrease in the photosynthetic rate in response to heat stress in both the cvs. HD2985 and HD2329. Decrease in photosynthetic rate was observed more in the thermotolerant cultivar (HD2985) at both stages, as compared to thermosusceptible cultivar HD2329 (Fig. 3). Percentage decrease in photosynthetic rate (P_i) of HD2329 was significantly lower, as compare to HD2985. Farooq *et al.*, (2011) also reported decrease in the photosynthetic capacity of the plant under HS. Kumar *et al.*, (2016) and Chen *et al.*, (2017) recently

reported the decrease in photosynthetic rate in wheat in response to heat stress and they also correlated it with activity of various antioxidant enzymes.

Guaiacol peroxidase (GPX) activity assay

We observed an increase in guaiacol peroxidase (GPX) activity in both the cvs. and with successive treatments. The activity was observed more in thermotolerant cv. HD2985 than thermosusceptible HD2329. In both the stages *i.e.* pollination and grain-filling, the activity was higher with successive treatment of HS in both the cultivars. In HD2985, the specific activity of Guaiacol peroxidase (GPX) was observed maximum (168.29 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein) at 38°C for 1 h (T_2) and minimum of 95.81 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein in control at pollination stage. During grain-filling stage, maximum Gpx of 178.59 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein was observed in response to 38°C for 1 h (T_2) and 118.59 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein in control (Fig. 4). HD2329 showed maximum activity of 110.74 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein and a minimum of 85.93 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein at pollination stage. During grain-filling, maximum of 151.1 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein and minimum of 97.63 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein was observed in response to 38°C for 1 h (T_2) and control, respectively. Chen *et al.*, (2017) also reported increase in the GPX activity in response to heat stress in wheat.

Table.1 List of primers used for the cloning and expression analysis of oxygen evolving enhancer protein gene identified from wheat using de novo assembly

primers	Primer sequence (5'-3')	Tm (°C)
PsbO-f	ATGGCAGCGTCTCTCCAAG	59.5
PsbO-r	CTAGTTAGACTCGAGCTGCG	60.5
qPsbO-f	CCAGAGCAAGACCTACATGG	58
qPsbO-r	CCATCTCGTCAAGGGTGTAG	58
Act-f	GCGGTGCGAACAAGTGGTATT	58.4
Act-r	GGTCCAAACGAAGGATAGCA	58.4

Fig.1a Conserved domain prediction of putative *PsbO* gene cloned from HD2329 cv. of wheat using Conserved Domain search tool of NCBI (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>)

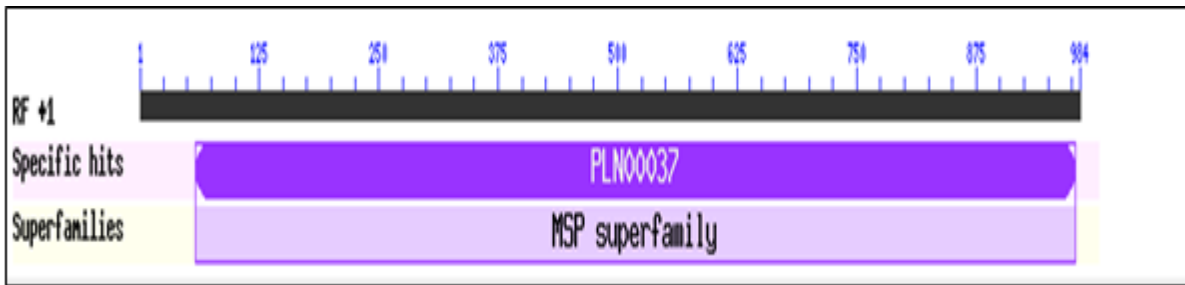
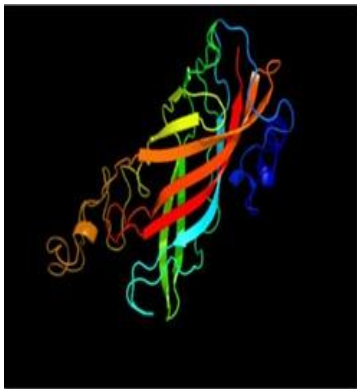
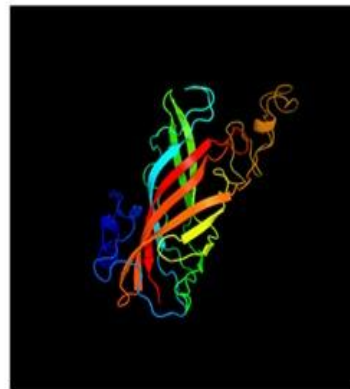


Fig.1b Predicted protein model of putative cloned *PsbO* gene from wheat cv HD 2329



a) Protein structure of *PsbO* gene



b) Structure of *PsbO* protein reported from *spinacia oleracea* which was u prediction. (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>)

Fig.1c Predicted phosphorylation site on putative *PsbO* gene cloned from HD2329 cultivar of wheat using NetPhos 3.1 (<http://www.cbs.dtu.dk/services/NetPhos/>)

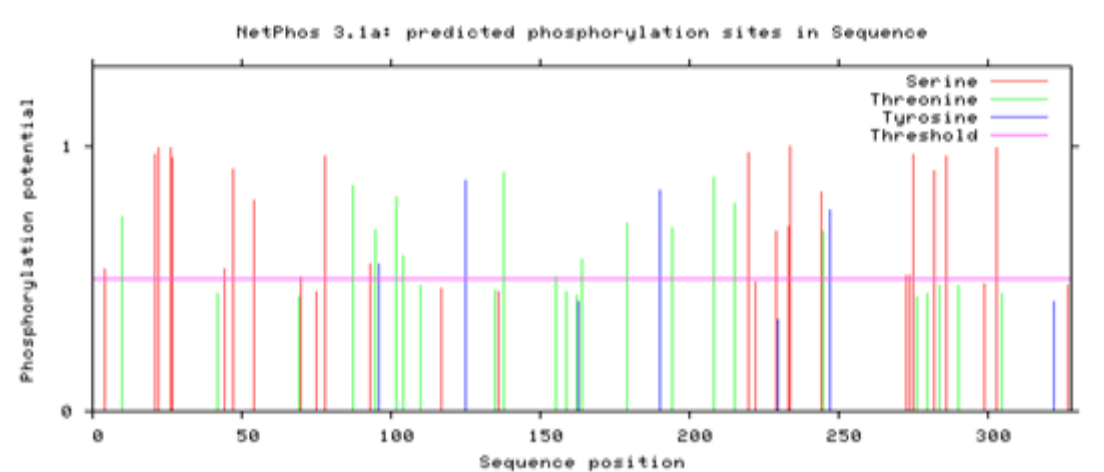


Fig.1d Ramachandran plot of predicted PsbO protein cloned from HD2329 cv. of wheat using RAMPAGE

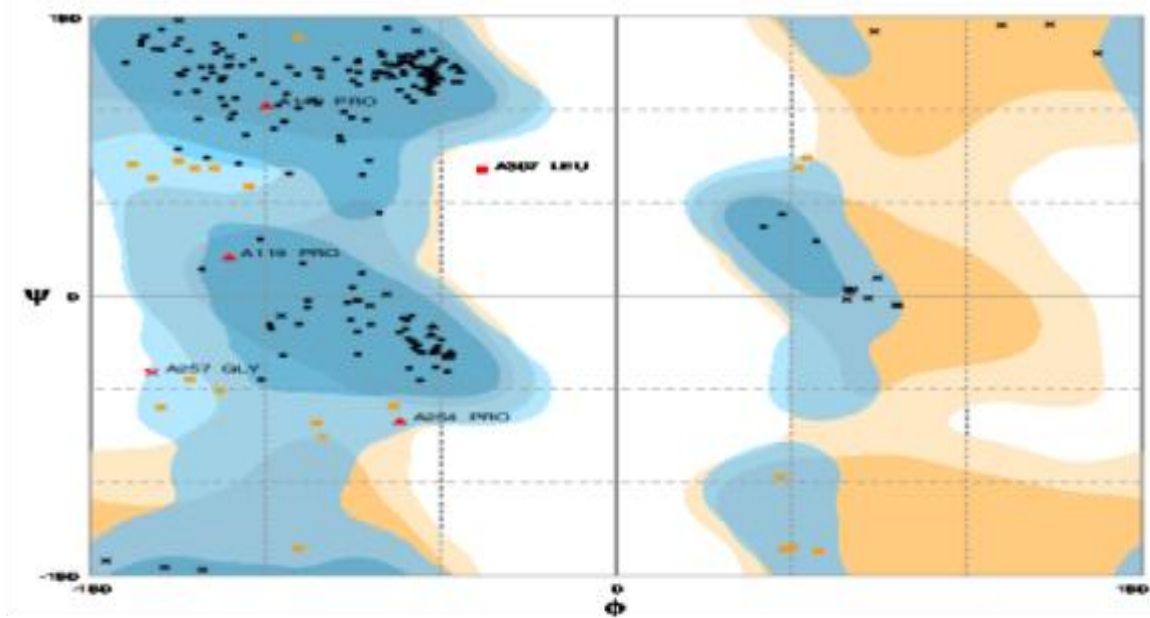


Fig.2 Expression profiling of putative PsbO gene transcript of wheat under differential heat stress at different stages; in thermotolerant cv. HD2985 and thermosusceptible cv. HD2329; C- 22°C; T1- HS of 30°C for 1hour, T2- HS for 38°C for 1 h; β -actin gene was used as endogenous gene control; Relative fold express was calculated by Pfaffl method (Pfaffl, 2002); Vertical bar indicates SE (n=3)

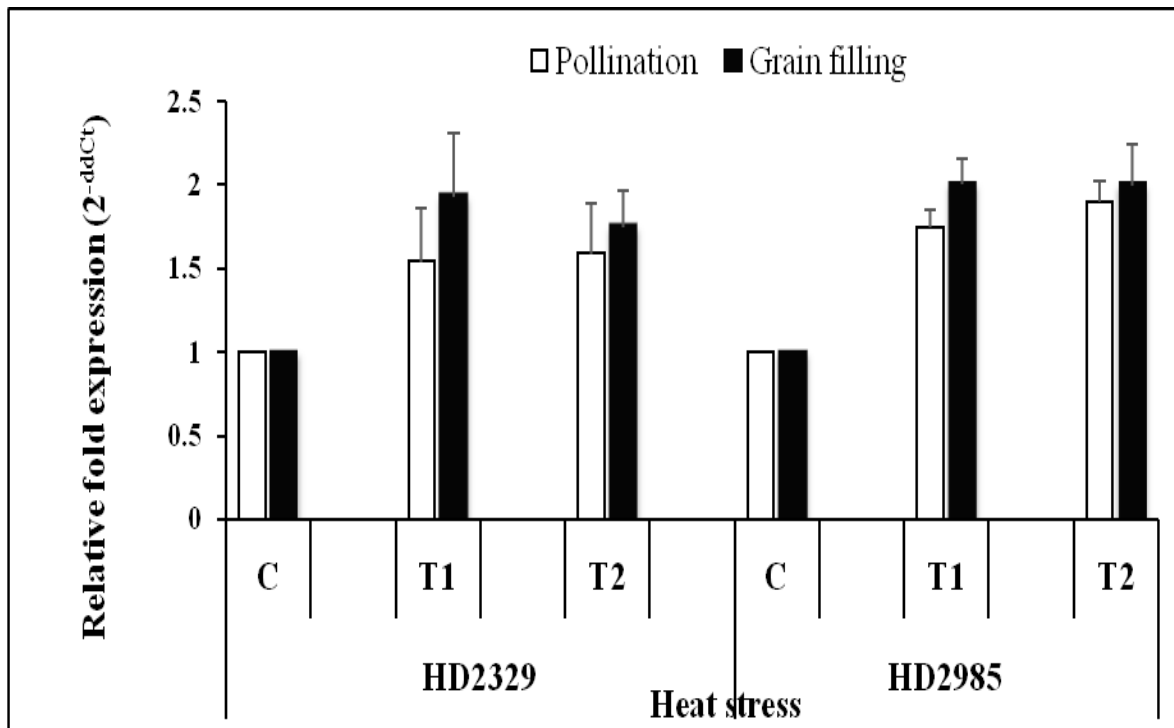


Fig.3 Photosynthetic rate of thermotolerant wheat cv. HD2985 and thermosusceptible cv. HD2329 at pollination and grain-filling stage under differential heat stress treatment; C-22°C; T1- HS of 30°C for 1 h, T2- HS for 38°C for 1 h; Vertical bar indicates SE(n=3)

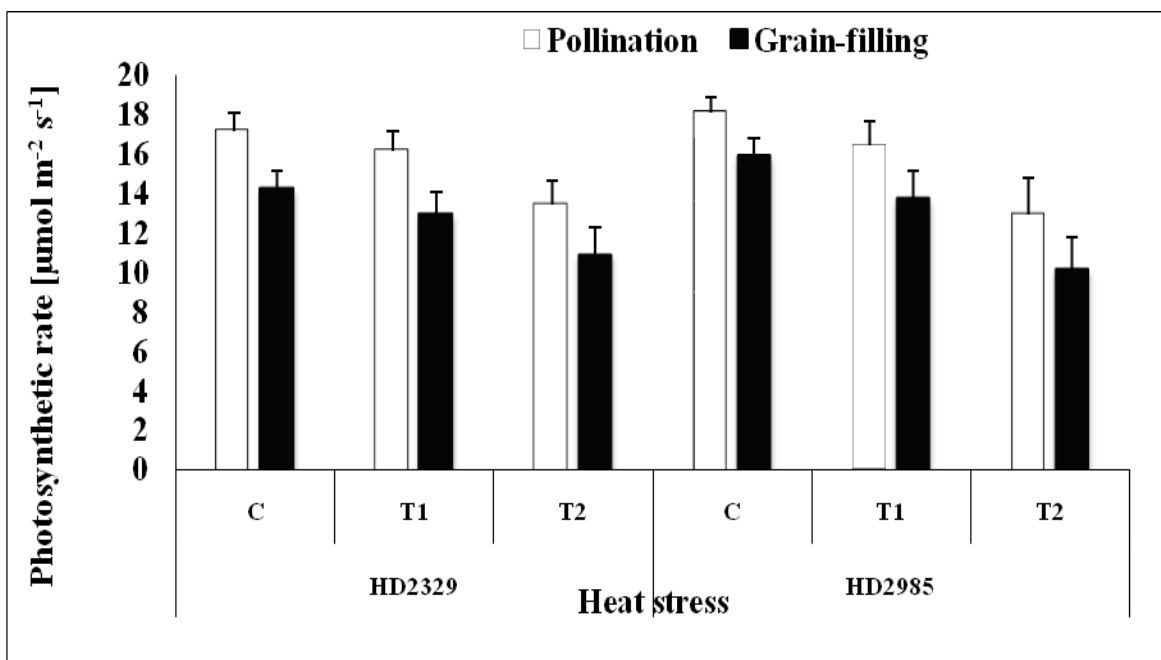


Fig.4 Guaiacol peroxidase (GPX) activity assay in thermotolerant wheat cv. HD2985 and thermosusceptible cv. HD2329 at pollination and grain-filling stage under differential heat stress treatment; C-22°C; T1- HS of 30°C for 1 h, T2- HS for 38°C for 1 h; Vertical bar indicates SE (n=3)

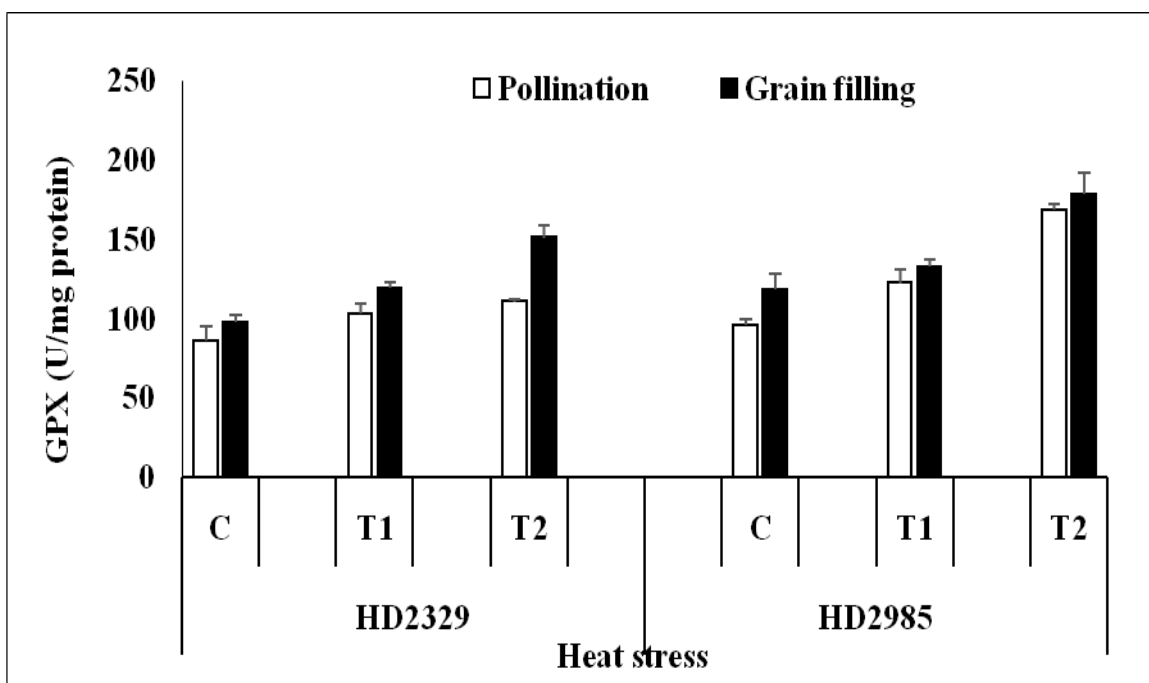


Fig.5 Superoxide dismutase activity assay in thermotolerant wheat cv. HD2985 and thermosusceptible cv. HD2329 at pollination and grain-filling stage under differential heat stress treatment; C-22°C; T1- HS of 30°C for 1 h, T2- HS for 38°C for 1 h; Vertical bar indicates SE (n=3)

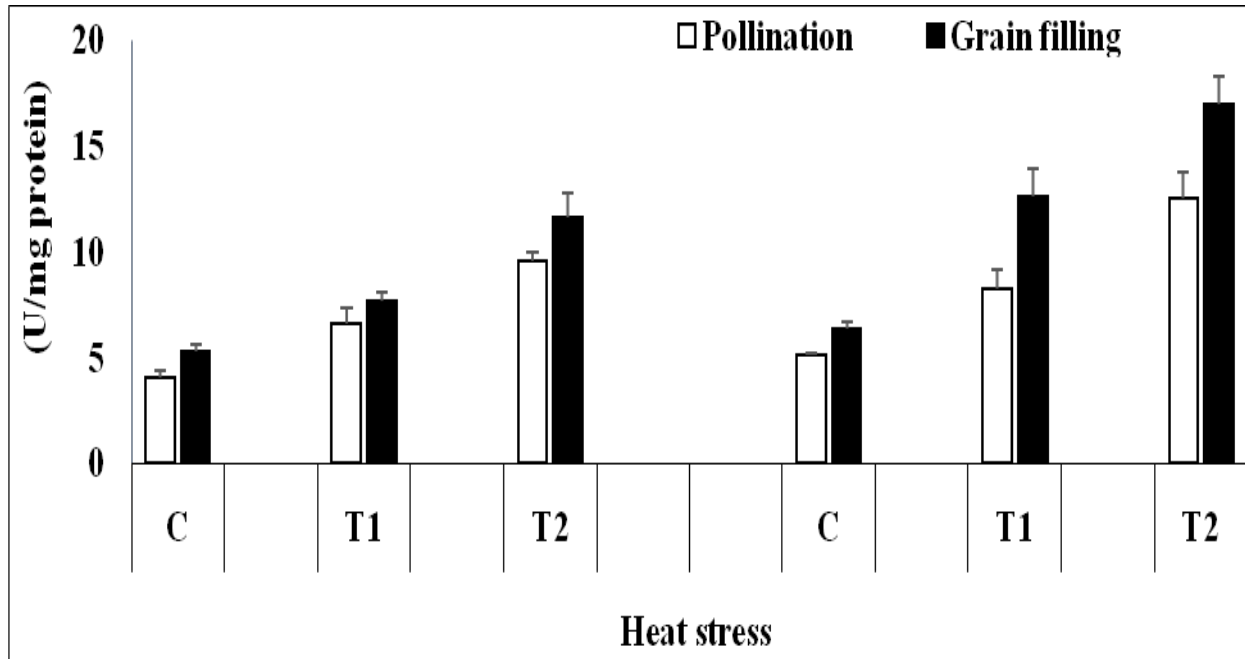


Fig.6 Catalase activity assay in thermotolerant wheat cv. HD2985 and thermosusceptible cv. HD2329 at pollination and grain-filling stage under differential heat stress treatment; C-22°C; T1- HS of 30°C for 1 h, T2- HS for 38°C for 1 h; Vertical bar indicates SE(n=3)

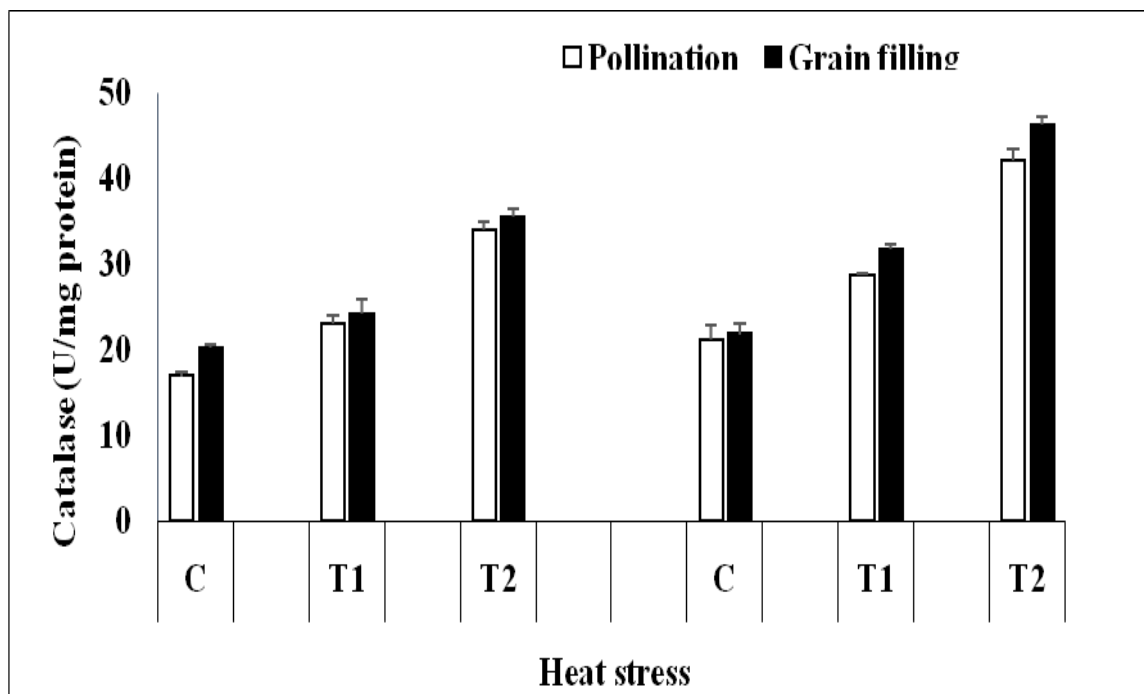
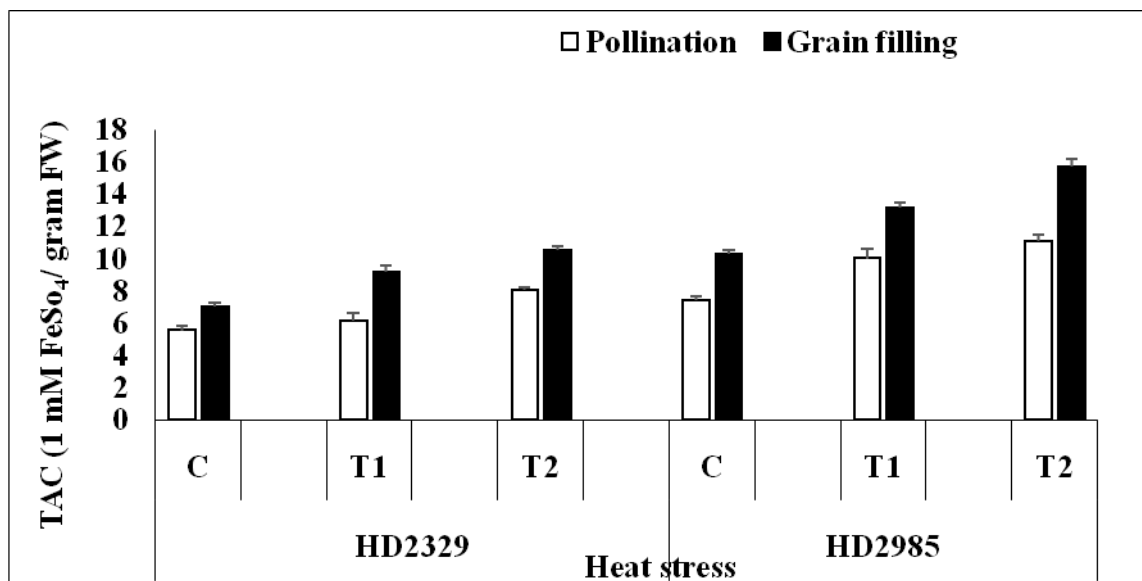


Fig.7 Estimation of Total Antioxidant Capacity (TAC) in thermotolerant wheat cv. HD2985 and thermosusceptible cv. HD2329 at pollination and grain-filling stage under differential heat stress treatment; C-22°C; T1- HS of 30°C for 1 h, T2- HS for 38°C for 1 h; Vertical bar indicates SE (n=3)



Superoxide dismutase activity assay

Superoxide dismutase (SOD) is one of important antioxidant enzyme for cellular defense. We observed a significant increase in SOD activity in all the treatment with respect to control in this study. We observed significant increase in SOD in the thermotolerant cultivar (HD2985) than thermo susceptible (HD2329) cultivar at both pollination and grain filling stages under the HS of 30°C and 38°C. HD2985 showed maximum SOD activity of 12.525 Umg⁻¹protein during pollination stage and 17.03 Umg⁻¹ protein at grain-filling in response to 38°C, 1 h. Similarly, under control condition, we observe SOD activity of 5.1 Umg⁻¹ and 6.35 Umg⁻¹ during pollination and grain-filling stages, respectively (Fig. 5). In thermo susceptible cultivar HD2329 the maximum of 9.555 Umg⁻¹ and 11.665 Umg⁻¹ was observed at 38°C, 1 h in pollination and grain-filling stages, respectively which is significantly higher than 30°C for 1h (T₁) and control (22°C). The observation is in conformity with

the findings of He *et al.*, (2005) and Kumar *et al.*, (2012).

Catalase activity assay

We observed an increased in catalase activity in both the cultivar and with successive treatment. The activity was observed more in thermotolerant cultivar *viz.* HD2985, than thermosusceptible HD2329. In both the stages of development, the activity was higher with successive treatment of heat stress in both the cultivar. The specific activity of catalase (CAT) was observed maximum of 42.15 μmol min⁻¹ mg⁻¹ protein to HS of 38°C for 1 h (T₂) and minimum of 21.15 μmol min⁻¹ mg⁻¹ protein in control condition at pollination stage whereas a maximum of 46.3 μmol min⁻¹ mg⁻¹ protein to HS of 38°C for 1 h (T₂) and 21.8 μmol min⁻¹ mg⁻¹ protein at control in grain filling stage of HD2985 (Fig. 6). In thermosusceptible cultivar, HD2329 maximum activity of 34.05 μmol min⁻¹ mg⁻¹ protein and a minimum of 16.9 μmol min⁻¹ mg⁻¹ protein at pollination stage and a

maximum of 35.45 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein and 20.2 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein in grain filling at 38°C (HS for 1 h) and control respectively. Almeselmani *et al.*, (2006) also showed the higher activity of antioxidant enzymes under heat stress in wheat.

Total antioxidant capacity

TAC is a measure of the antioxidant potential of the tissues, and is one of the important biochemical parameters used for assessing the thermotolerance of crops. We observed significant increase in TAC in the thermotolerant (HD2985) than thermosusceptible (HD2329) cultivar at both pollination and grain filling stages under HS of 30°C and 38°C. In HD2985 a maximum of 11.055 mmol g^{-1} FW at pollination and 15.67 mmol g^{-1} FW at grain filling at 38°C was observed in comparison to 7.42 mmol g^{-1} FW, 10.26 mmol g^{-1} FW in control at pollination and grain-filling stage respectively. In thermo susceptible cv. HD2329, the maximum of 8.03 mmol g^{-1} FW and 10.54 mmol g^{-1} FW was observed at 38°C in pollination and grain-filling stage, respectively which is significantly higher than 30°C for 1h (T₁) and control(22°C) (Fig. 7). Increase in the TAC under HS was observed more in thermotolerant cv. The finding is in agreement with the observation of Sairam *et al.*,(2002) who explained the activities of the antioxidant enzymes involved in thermotolerance goes up under HS; similar observations were reported from our lab also (Kumar *et al.*, 2013 and 2014; de Leonardis *et al.*, 2015).

Heat stress has a severe detrimental effect on wheat. As photosynthesis is the most vulnerable physiological process towards heat, photosystem II act as primary point heat response. In this study, we identified and cloned major extrinsic protein of photosystem II *i.e.* PsbO (transcript _CDS_52998_unigene

_92869) from HD2329. PsbO was observed to have higher expression in thermotolerant, as compared to thermosusceptible wheat cvs. A positive correlation of *PsbO* expression was observed with the photosynthetic rate and antioxidant enzymes (GPX, SOD, CAT and TAC) at different stages of wheat growth. There is a need to explore many more PsbO gene in cereals in order to manipulate the carbon assimilatory process under HS.

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