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Effects of Prolonged High Temperature Stress on Respiration, Photosynthesis and Gene Expression in Wheat (*Triticum aestivum* L.) Varieties Differing in their Thermotolerance

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ABSTRACT

High temperature (HT) stress is a major environmental stress limiting wheat productivity worldwide. This study was conducted to analyze the effect of prolonged HT on wheat genotypes C306 (HT tolerant) and PBW343 (HT susceptible) in controlled environment chambers. After an initial 45-days growth at 23/18°C day/night, one set of plants (control) was maintained at the same conditions, while the other set of plants was exposed to HT (35/25°C day/night). Effect of heat stress was analyzed after one week (vegetative), 30-days (anthesis) and 45-days (15 days after anthesis) of HT. HT significantly reduced the leaf chlorophyll (chl) content, photosynthetic rate and Rubisco activity in both the genotypes at all the growth stages. However, the reduction was more in heat-susceptible genotype PBW343 than heat-tolerant genotype C306. C306 showed higher respiratory homeostasis than PBW343, under prolonged heat stress. Heat tolerant C306 showed less reduction in photosynthesis and maintained respiration, while heat susceptible PBW343 showed higher reduction in photosynthesis accompanied by significant increase in respiration. Better tolerance in photosynthesis to long-term heat stress in C306 can be attributed to less reduction in total chl and chl *b* content and Rubisco activity. Maintenance of Rubisco activity in C306 under HT may be due to maintenance of expression of *rbcS* and *rbcL* genes. Maintenance of high expression levels of *HSP101* during long-term heat stress in C306 may also be reason for better tolerance of C306 as compared with PBW343, as HSP101 is crucial for both basal and acquired thermotolerance.

Keywords: heat stress, *HSP101*, photosynthesis, Rubisco
Abbreviations: chl, chlorophyll; **HSP**, heat shock protein; **HT**, heat stress; **LHC**, light harvest complex; **PsbA**, 32KDa D1 protein of PSII; **PsbO**, oxygen evolving complex 33 KDa protein; **PSI**, photosystem I; **PSII**, photosystem II; ***rbcL***, genes coding for Rubisco large subunit; ***rbcS***, genes coding for Rubisco small subunit

INTRODUCTION

High temperature (HT) stress adversely affects crops productivity, particularly wheat, which is sensitive to stress injury. This crop is often grown in areas where HT considered major constraint for growth and yield (Almeselmani *et al*. 2006, 2009a; Efeoglu and Terzioglu 2009). Continual HT stress is a problem in 7 million ha of wheat cropped area, while terminal HT stress is common in 40% of the irrigated wheat growing areas of the world (Fischer and Byerlee 1991). Yield reduction in wheat under HT stress is caused by accelerated phasic development (Warrington *et al*. 1977; Rawson and Bagga 1979), reduction in photosynthesis (Conroy *et al*. 1994; Almeselmani *et al*. 2009b; Almeselmani 2010), accelerated senescence (Kuroyanagi and Paulsen 1985; Almeselmani 2010), increase in respiration (Berry and Bjorkman 1980) and inhibition of starch accumulation in developing kernels (Jenner 1994; Chinnusamy and Khanna-Chopra 2003).

Heat stress affects the metabolism and structure of plants, especially cell membranes and many basic physiological processes such as photosynthesis, respiration and water relations (Wahid *et al*. 2007). Reduction of photosynthesis by HT stress appears to be a major factor that imposes source limitation to yield formation. Calvin cycle reactions and photosynthetic electron transport are susceptible to HT stress (Berry and Björkman 1980). Photosystem II (PSII) complex is thermally labile and is considered as the most heat sensitive component of the electron transport chain (Havaux and Tardy 1996; Yamane *et al*. 2000; Almeselmani *et al*. 2009b). In spinach, HT causes cross-linking of D1 protein with D2 protein and thus leads to dramatic decrease of PSII yield (Ohira and Yamamoto 2001). In plants, *psbO* (gene encoding the oxygen evolving complex 33 KDa protein) encodes one of the most important extrinsic proteins for oxygen-evolving complex (OEC). PsbO has a stabilizing effect on the Mn cluster where water oxidation occurs. Hence, PsbO is often referred to as the Mn-stabilising protein (Heredia and de las Rivas 2003). The OEC of photosystem II (PSII) is susceptible to heat stress as heat stress releases two of four Mn atoms from the catalytic site of the oxygen-evolving machinery, and thus inactivates the OEC (Nash *et al*. 1985). However, the effect of HT stress on the expression of PSII genes such as *psbA* and *psbO* has not been examined.

Calvin cycle reactions are more sensitive to heat stress than PSII electron transport. The primary site of inhibition of Calvin cycle under HT appears to be the Rubisco activation via Rubisco activase in wheat (Law and Crafts-Brandner 1999). As temperature increases Rubisco activase becomes less effective in maintaining Rubisco in a catalytically active state (Salvucci *et al*. 2004). At HT Rubisco activase denatures forming insoluble aggregates that are incapable of removing inhibitor, consequently a high activation state of Rubisco cannot be maintained (Sage *et al*. 2008). Decrease in Rubisco activation accompanied by increase in leaf temperatures is closely correlates with the extent of photosynthetic inhibition (Law and Crafts-Brandner 1999; Crafts-Brandner and Salvucci 2000, 2002). Moreover, heat stress decreases the expression levels of

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Rubisco genes. Rubisco small subunit (rbcS) synthesis is more susceptible to heat stress than Rubisco large subunit (rbcL) in field-grown soybean (Nover *et al*. 1989). Genetic differences in the heat-tolerance of Rubisco synthesis have been found even within a species. At 34°C, the heat tolerant wheat variety 'Mustang' was able to maintain higher level of rbcS synthesis than heat-susceptible variety 'Sturdy' (Krishnan *et al*. 1989). However, after one-hour heat acclimation at 37°C, these two wheat varieties did not show any difference in SSU synthesis. This may be due to protection of transcriptional and/or translational machinery by heat shock proteins (HSPs) induced during heat acclimation (Krishnan *et al*. 1989).

Prokaryotic and eukaryotic respond to different biotic and abiotic stresses by inducing the synthesis of protein family called stress proteins. HSPs have been known to protect cells against deleterious effects of stress (Efeoglu 2009). Al-Whaibi (2011) indicated that HSPs protect cells from injury and facilitate recovery and survival after a return to normal growth conditions. HSPs may confer thermotolerance by protection of nucleus, ribosome (Schlesinger 1990), protein synthesis (Krishnan *et al*. 1989), thylakoid membranes of chloroplast (Schuster *et al*. 1988) and electron transport chain of mitochondria (Chou *et al*. 1989) and chloroplasts (Heckathorn *et al*. 1998) under heat stress. HSPs act as molecular chaperones that counteract protein denaturation and aggregation under heat stress (Jin *et al*. 1989; Lee *et al*. 1995) and reactivate/repair heat damaged proteins (Mummert *et al*. 1993; Lee and Vierling 2000), and target non-native proteins for degradation by interacting with ubiquitin and certain proteases (Parsell and Lindquist 1993). Genetic evidences and transgenic analyses of HSP100 family members, *E. coli ClpB* (Squires *et al*. 1991), yeast *HSP104* (Sánchez and Lindquist 1990) and *HSP101* of *Arabidopsis* (Queitsch *et al*. 2000; Hong and Vierling 2001) and maize (Nieto-Sotelo *et al*. 2002) showed that HSP101 is essential for acquired as well as basal thermotolerance. Yeast HSP104 (Parsell *et al*. 1994) and rice HSP101 (Agarwal *et al*. 2003) mediate the resolubilization of heat-inactivated proteins from insoluble aggregates. Transgenic *Arabidopsis* plants overexpressing *HSP101* tolerated sudden shifts to extreme temperatures better than did vector controls, while antisense or co-suppression of *HSP101* led to heat susceptibility (Queitsch *et al*. 2000). Transgenic rice plants overexpressing *AtHSP101* also showed significantly better growth performance in the recovery phase following the heat stress (Katiyar-Agarwal *et al*. 2003). Thus, HSP101 is pivotal for thermotolerance across organisms. However, the expression of *HSP101* under prolonged heat stress in wheat has not been examined.

Often the effect of HT stress on gene expression is examined after short durations (minutes to days) of heat stress. However under field conditions, wheat crop experiences prolonged heat stress (days to months), i.e., from sowing to harvesting under continual heat stress environments, and during the grain filling period under terminal heat stress. Hence this study was conducted to analyze the effect of long-duration HT stress (7 days to 45 days) on photosynthesis, respiration and expression of *rbcL*, *rbcS*, *psbA*, *psbO* and *HSP101* genes in a heat tolerant wheat genotype C306 (Khanna-Chopra and Viswanathan 1999; Almeselmani *et al*. 2006) and a heat susceptible wheat genotype PBW343 (Almeselmani *et al*. 2006) under controlled environment conditions. The factors contributing stability in photosynthesis under long term heat stress was examined in terms of Rubisco activity, expression of Rubisco genes and genes encoding two important proteins of PSII namely psbA and psbO.

MATERIALS AND METHODS

Plant culture and treatments

Two wheat genotypes namely, 'C306' and 'PBW343' were grown in controlled environment growth chambers at the National Phytotron Facility, Indian Agricultural Research Institute, New Delhi. Wheat seeds were sown in pots (15 cm diameter and 30 cm height, media consisting of 2: 1: 1 coco-coir peat: vermiculite: sand), irrigated with deinonized water until germination, ¼-strength Hoagland and Arnon (1950) solution from germination to 2-3 leaf stage and then with full-strength Hoagland and Arnon solution. In the control treatment, growth chamber temperatures were maintained at 23/18°C day/night (control) through out the experiment, while in the other growth chamber, temperatures were maintained at 23/18°C day/night till maximum tillering stage (45 days after germination), after that temperatures were raised to 35/25°C day/night to impose heat stress treatment. On the first day of HT, growth chamber temperature was raised gradually at a rate of approximately 1°C/h till it attained treatment temperatures, and maintained at that temperature for the rest of the treatment duration. Later on each day the plants were directly exposed to 35/25°C day/night cycle. In both treatments a 14-h photoperiod at 250 μmol $m^{-2}s^{-1}$ was applied.

Physiological analysis

The rate of leaf photosynthesis and respiration were measured by using an infra-red gas analyzer (LiCOR 6200, LI-COR Inc., Lincoln, NE) at three stages, namely, vegetative stage (7 days after HT treatment), anthesis (30, 35 days after HT treatment in 'PBW343' and 'C306', respectively) and 15 days after anthesis (45 and 50 days after HT treatment in 'PBW343' and 'C306', respectively). At the same dates leaf samples were collected from treated and control plants, and used immediately to measure leaf chlorophyll (chl) content and assay Rubisco activity. For chl estimation, the upper most fully expanded leaf at vegetative stage and flag leaf at reproductive stages (anthesis and 15 days after anthesis) were sampled. Leaf tissue (0.1 g) was incubated in 10 mL dimethyl sulphoxide at 65°C for 4 h, after which the absorbance of chl extract was measured at 663 and 645 nm (Hiscox and Israelstam 1979). Leaf tissue (0.5 g) was ground in a mortar with 10 mL ice chilled buffer solution containing 100 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 12.5% (v/v) glycerol and then centrifuged at 39,000 × *g* for 30 min. The supernatant was used for the estimation of soluble protein content and Rubisco activity. Soluble protein content of leaf samples was determined by the method described by Lowry *et al*. (1951). Rubisco activity was determined by a radiochemical method (Nicco *et al*. 1993). For activation of Rubisco, 0.1 mL of crude enzyme extract was added into medium containing 30 μM Tris-HCl solution containing 20 μM $NaHCO_3$, 0.1 μM EDTA, 3.03 μM $MgCl_2$ and $NaH^{14}CO_3$ (specific activity 125.17 GBq mol^{-1}) in liquid scintillation vials and incubated at 25°C for 10 min. The reaction was initiated by the addition of 0.2 mL of 0.2 mM ribulose bis-phosphate solution to the assay vials and incubated at 25°C in a continuous shaking water bath for 5 min. The reaction was terminated by adding 0.2 mL of 2 M HCl to each vial. Then the vials were left open in a hood for 12 h. The incorporation of ^{14}C into acid-stable product was measured after adding 4 mL of scintillation cocktail to each vial and the radioactivity was measured by using a liquid scintillation counter (Packard TRICARB-Model 1600 TR, Meriden, USA).

RNA isolation and gene expression analysis

Leaf samples were collected at the same three stages for RNA isolation, and analyzed by RT-PCR and Northern blotting. Immediately after sampling the leaves were frozen in liquid N_2 and stored at –80°C. Total RNA was extracted from the frozen leaf tissue using TRIZOL reagent (Invitrogen). Leaf sample (1 g) was ground in liquid nitrogen, and the powder was added in to 10 mL of TRIZOL reagent in an RNAse-free centrifuge tube and incubated at room temperature for 5 min with intermittent vortexing. After adding 2 mL of chloroform, the tubes were shaken for 15 s. After 5 min of incubation at room temperature, the tubes were centrifuged at 12,000 × *g* at 4°C for 15 min. The resulting upper aqueous colorless phase was transferred to a new tube, to which 5 mL of isopropanol was added. The contents of the tubes were mixed well, incubated for 10 min at room temperature and then centrifuged at 12,000 × *g* at 4°C for 30 min. The resulting pellet was

washed with 75% (v/v) ethanol, and the tubes were respun at 10,000 × *g* at 4°C for 5 min. After removing the ethanol, the pellet was air-dried for 10 to 15 min at room temperature. The RNA was resuspended in DEPC-treated water. To eliminate DNA from aqueous RNA extractions, samples of isolated nucleic acid were treated with 10 units of RNase-free DNase I (Qiagen, USA). Total RNA was quantified spectrophotometrically by measuring absorbance at 260 nm. RNA was fractionated on 1% agarose gel to check the quantity and integrity.

Reverse transcription–polymerase chain reaction (RT-PCR) was used to study the effect of HT stress on the expression of *rbcS* and *rbcL* genes. One step RT-PCR (Qiagen, USA) was performed in a 50 µL reaction mixture containing 500 ng of total RNA in 0.2 mL thin walled tubes using thermal cycler (Perkin Elmer). For RT-PCR, the following gene-specific primers were designed based on the GenBank accession numbers AB042069 and AB042240 (*Triticum aestivum* chloroplast DNA) for *rbcS* and *rbcL*, respectively: *rbcL* –Forward primer: 5′-CGC CTC ATG GTA TCC AAG TTG-3′; *rbcL* –Reverse primer: 5′-CGA TTA GCT GCT GCA CCA GGT G-3′; *rbcS* –Forward primer: 5′-CGT GAT GGC TTC GTC GGC TAC-3′; *rbcS* –Reverse primer: 5′-CTG AAT GCG ATG AAG CTG AC-3′.

The amplified products were analyzed by electrophoresis on a 1.4% agarose gel containing 0.5 µg/mL ethidium bromide.

Probes for *psbA*, *psbO* and *HSP101* were amplified from wheat var. 'C306' by RT-PCR with gene-specific primers. The following gene-specific primers were designed based on the GenBank accession numbers AB042240 and X57408 for *psbA* and *psbO*, respectively: *psbA* – Forward Primer: 5′-GGA AGC TGC ATC TGT TGA TG-3′; *psbA* – Reverse Primer: 5′-CAA GGT TAG CAC GGT TGA TG-3′; *psbO* – Forward Primer: 5′-GAC CTT CGA CGA GAT CCA GA-3′; *psbO* – Reverse Primer: 5′-CCC TGG ATC TTG ACA TCC TT-3′.

We RT-PCR amplified (Forward Primer: 5′-CTT CGA CGA GGT TGA GAA GG-3′, Reverse Primer: 5′-CTG GAT CAG GAT GTC GGA CT-3′) partial 3′ coding sequence of *TaHSP101c*, members of HSP101/ClpB family and used it as probe in Northern analysis (GenBank Acc. No. AF174433; Campbell *et al*. 2001). This region of TaHSP101c showed significant sequence identity (85% identity with 1.6% gap) with *TaHAP101b* (GenBank Acc. No. AF097363; Campbell *et al*. 2001) and *TaHSP101* (GenBank Acc. No. AF083344; Wells *et al*. 1998). Hence, the probe used in this study can detect all three HSPs.

For Northern blot analysis, for each genotype equal amount of total RNA (20 µg) from normal and HT treated plants were fractionated on a 1.2% (w/v) denaturing formaldehyde-agarose gel as described by Sambrook *et al*. (1989). After electrophoresis, RNA was capillary-blotted to nylon membrane (Hybond-N, Amersham, Arlington Heights, IL) overnight using 10X SSC. Membranes were UV-crosslinked using a Stratalinker (Stratagene) and prehybridized in 200 mM Na_2PO_4, pH 7.2, 5% (v/v) SDS, 1 mM EDTA, 10 mg/mL bovine serum albumin, 0.1 mg/mL sheared salmon sperm DNA for 4 h at 65°C. Radio-labeled probes were prepared by using HexaLabel™ DNA labeling kit (MBI Fermentas). Blots were probed with denatured ^{32}P-labeled probes added directly to the prehybridization solution at 65°C for 16 h. Blots were washed twice for 15 min at 65°C with 40 mM Na_2PO_4, pH 7.2, 5% (v/v) SDS, 1 mM EDTA, washed once for 15 min at 65°C in 40 mM Na_2PO_4, pH 7.2, 1% (v/v) SDS, 1 mM EDTA and signals were detected by exposure to Kodak X-ray films (Sigma).

The relative expression levels were calculated from RT-PCR agarose gels for *rbcL* and *rbcS*, and from autoradiograms for *psbA*, *psbO* and *HSP101*. The images were first converted in to 8-bits gray scale image and then quantified by using AlphaImager® Imaging System (Alpha Innotech Corporation, San Leandro, CA 94577).

Statistical analysis

Data in each experiment were collected in triplicates. The analysis of variance (ANOVA) was done with two-factor (genotype and temperature) factorial analysis program of CIMMYT MSTAT program and the critical difference (CD) value for genotype × temperature treatment was calculated at $P = 0.0$đ.

RESULTS AND DISCUSSION

The optimum temperature for growth and yield of wheat is about 18–24°C. Even short periods (4–6 days) of very HTs (35-40°C) significantly decrease grain yield (Stone and Nicolas 1994, 1995). In many parts of wheat grown area of India, wheat crop experiences 35°C during grain development, and thus results in low productivity. Hence in this study, temperatures of 23/18°C and 35/25°C day/night were chosen for normal (control) and HT stress treatments, respectively.

HT stress significantly reduced leaf chl content in both genotypes at all three stages of growth. Heat stress reduced leaf chl content by 22.8 and 47.5% in 'C306' at anthesis and 15 days after anthesis respectively, while in PBW343, 29.2 and 60.9% reduction in chl content at anthesis and 15 days after anthesis, respectively, was observed under HT as compared with normal temperature (**Fig. 1A**). Reduction in chl content due to heat stress has been reported (Almeselmani *et al*. 2006, 2009a). Previous studies have shown that heat stress reduces chl content in wheat was due to inhibition of porphobilinogen deaminase activity and thus reduction in protochlide content in wheat seedlings upon exposure to short duration of heat stress at 42°C (Tewari and Tripathy 1998). A significant genotypic difference observed in heat stress-induced reduction in total chl content between 'C306' and 'PBW343' (**Fig. 1A**) suggests differential sensitivity of chl synthesis/breakdown to heat stress in these genotypes. Chl *b* is one of the light harvesting pigments that function to bring photons to the reaction centers, and it binds and stabilizes many of the light-harvesting complex (LHC) proteins. Chl *b* is more in light harvesting complex II (LCHII) of PSII as compared with PSI. In this study chl *a* and *b*

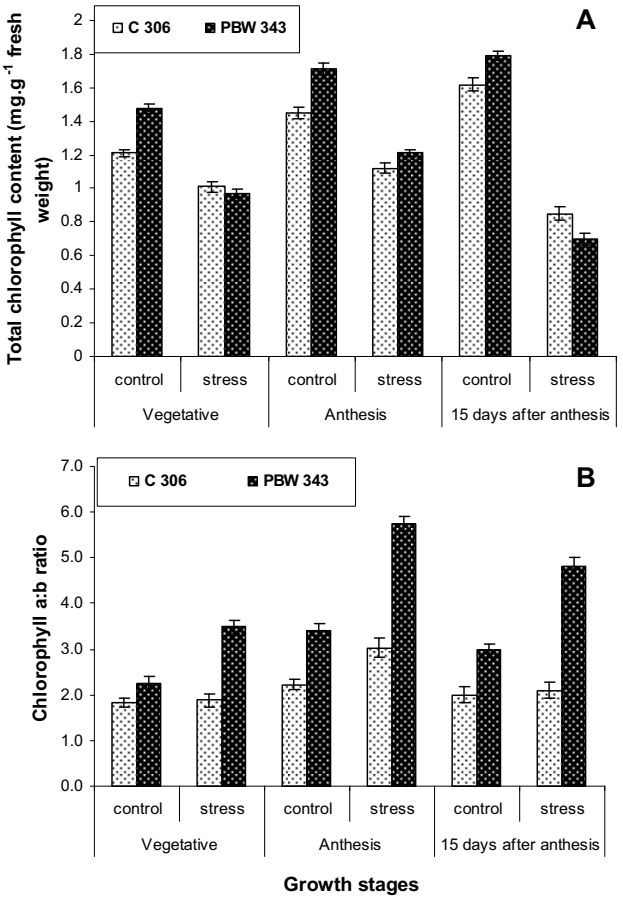

Fig. 1 Total chlorophyll content and chlorophyll *a*:*b* ratio in C306 and PBW 343 under normal temperature (25/18°C) and high temperature (35/25°C) at vegetative stage, anthesis and 15 days after anthesis. (**A**) CD at $P < 0.05$ for genotype × temperature interaction = 0.33. (**B**) CD at $P < 0.05$ for genotype × temperature interaction = 1.30. Vertical bars indicate SE, n = 3.

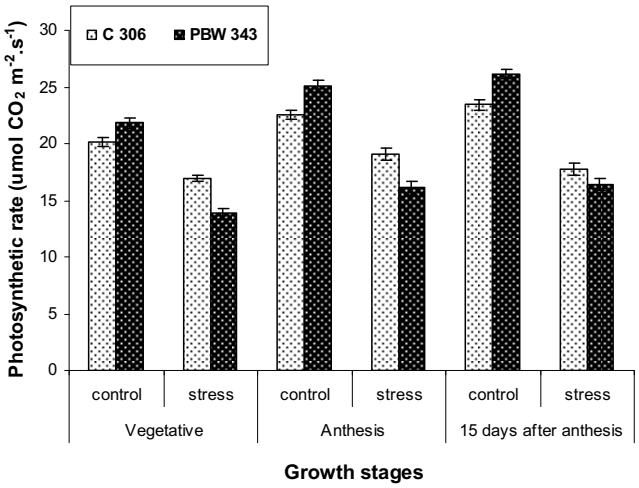

Fig. 2 Photosynthetic rate in C306 and PBW 343 under normal temperature (25/18°C) and high temperature (35/25°C) at the vegetative stage, anthesis and 15 days after anthesis. Vertical bars indicate SE, n = 3; For genotype × temperature interaction CD at $P < 0.05 = 3.32$.

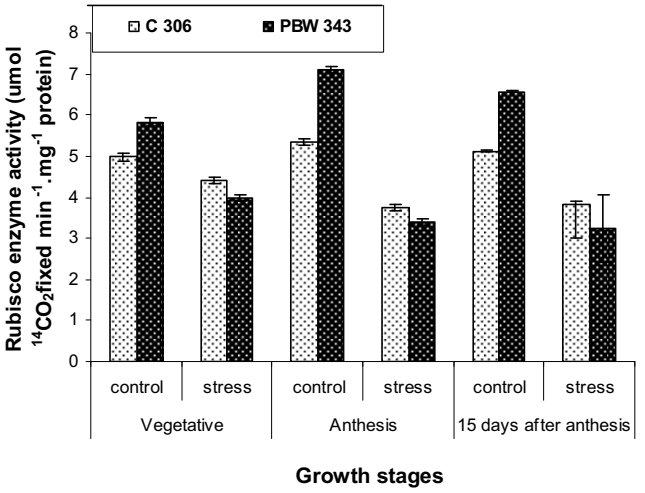

Fig. 3 Rubisco enzyme activity in C306 and PBW 343 under normal temperature (25/18°C) and high temperature (35/25°C) at vegetative stage, anthesis and 15 days after anthesis. Vertical bars indicate SE, n = 3; For Genotype × temperature interaction CD at $P < 0.05 = 1.31$.

were measured to study the effect of heat stress on chl *a/b* ratio. The reduction in chl *b* was more compared to chl *a* under heat stress and thus the chl *a/b* ratio increased under HT in both genotypes. However the chl *a/b* ratio was higher in 'PBW343' than that of 'C306' under heat stress, indicating the hypersensitivity of chl *b* formation/break down in 'PBW343' than that of 'C306' to long-term HT (**Fig. 1B**). A decreased chl *a/b* ratio can be the result of reduction in LCHII antenna pigments or a reduction of the PSII-to-PSI ratio (Montane *et al*. 1998). Prolonged heat stress mediated reduction in total chl and chl *b* content is more in heat susceptible 'PBW343' as compared with 'C306', and thus maintenance of higher LCHII and the PSII: PSI ratio under long-term heat stress appears to be one of the reasons for higher photosynthesis in 'C306' (**Fig. 2**). Previous studies have shown significant correlation between heat-induced reduction in chl content and photosynthetic rate under short periods of heat stress (Mullarkey and Jones 2000). In our study also similar results were observed.

Photosynthesis is known to be one of the most heat sensitive processes and it can be completely inhibited by HT and these photosynthesis decreases could result from the inhibition of PSII activity, which has been shown to be the most thermally labile component of the electron transport chain (Camejo *et al*. 2005; Efeoglu and Terzioglu 2009). HT significantly reduced the leaf photosynthetic rate in both genotypes at all three stages of plant growth compared to their respective control plants. Although, the high yielding genotype 'PBW343' showed higher leaf photosynthesis rate under normal temperatures, the photosynthesis rates were superior in heat tolerant genotype 'C306' as compared with 'PBW343' under heat stress (**Fig. 2**). Even in the absence of heat stress injury, photosynthesis would be expected to decline as temperature increases because photorespiration increases with temperature faster than does photosynthesis (Sharkey 2005). We have measured the total Rubisco activity after incubating the crude enzyme in an activation buffer (Nicco *et al*. 1993). Heat stress reduced activity of Rubisco enzyme in both genotypes (**Fig. 3**). However, heat-tolerant 'C306' showed less reduction in Rubisco activity as compared with that of heat-susceptible 'PBW343' (**Fig. 3**). Previous studies have shown that total Rubisco activity was not affected by temperatures as high as 45°C, while even moderately HTs (>30°C) affect Rubisco activase-mediated activation of Rubisco in wheat (Feller *et al*. 1998). Rubisco activase is highly sensitive to heat stress in wheat and other plants (Law and Crafts-Brandner 1999; Crafts-Brandner and Law 2000; Crafts-Brandner and Salvucci 2000, 2002). Temperature-induced inhibition of Rubisco activation was fully reversible at temperatures below 40°C, while above that temperature formation of high-molecular-weight aggregates of activase due to thermal denaturation results in irreversible loss of Rubisco activase (Feller *et al*. 1998; Salvucci *et al*. 2001). In this study, significant difference in the Rubisco activity was observed between 'C306' and 'PBW343' after exposure to various periods of heat stress. The percent reduction in Rubisco activity was 11.4, 29.9 and 25.7 in 'C306', 31.7, 52.4 and 50.7 in 'PBW343' at one week (vegetative), 30 days (anthesis) and 45 days of HT (15 days after anthesis) respectively as compared with their respective controls (**Fig. 3**). This indicates the differential sensiti-

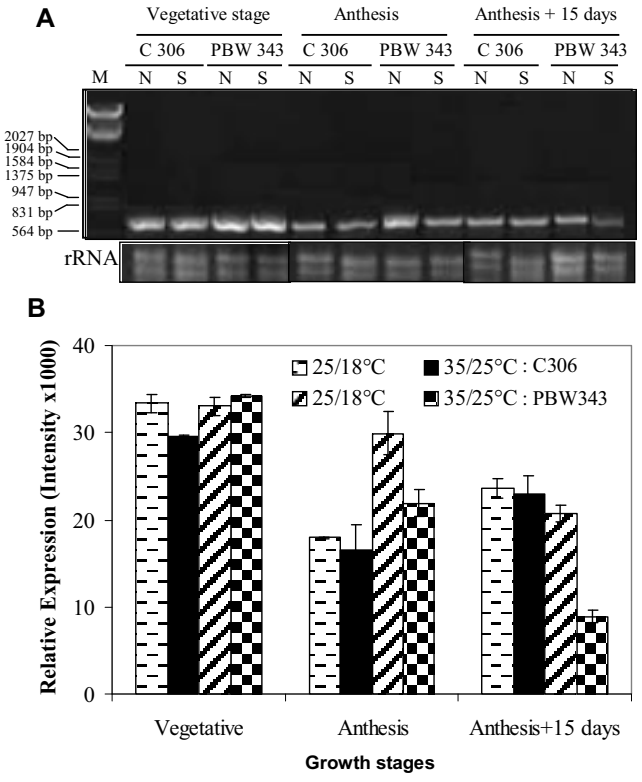

Fig. 4 Expression of Rubisco large subunit (*rbcL*) gene in response to high temperature stress. N, 25/18°C day/night; S, 35/25°C day/night; Plants were exposed to high temperature stress from 45 days after sowing till maturity. Leaf samples from control and high temperature (HT) treated plant were collected at the vegetative stage (7 days after HT), anthesis (30 days after HT) and 15 days after anthesis (45 days after HT). (**A**) RT-PCR of *rbcL*; (**B**) Densitometric quantification of relative expression levels of *rbcL*. M = λ *Hin*dIII/*Eco*RI marker.

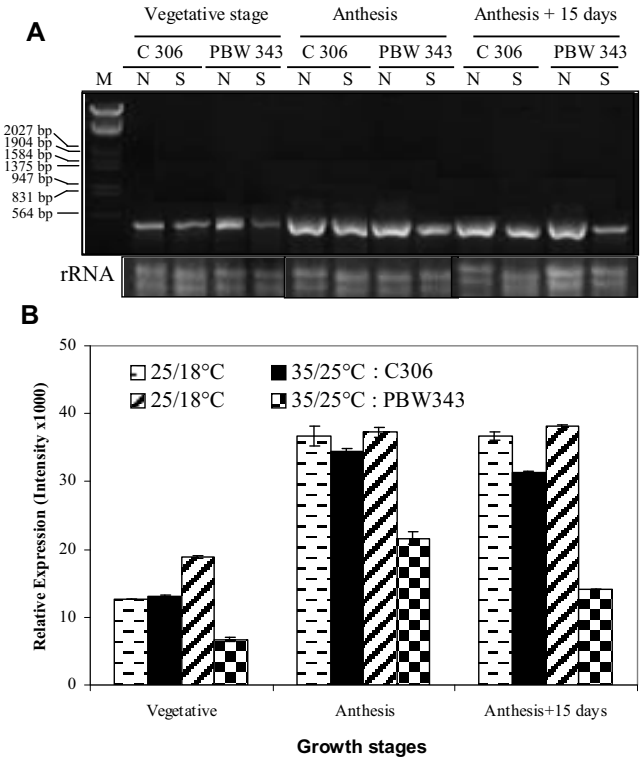

Fig. 5 Expression of Rubisco samll subunit (*rbcS*) gene in response to high temperature stress. N, 25/18°C day/night; S, 35/25°C day/night; Plants were exposed to high temperature stress from 45 days after sowing until maturity. Leaf samples from control and high temperature (HT) treated plant were collected at the vegetative stage (7 days after HT), anthesis (30 days after HT) and 15 days after anthesis (45 days after HT). (**A**) RT-PCR of *rbcS*; (**B**) Densitometric quantification of relative expression levels of *rbcS*. M = λ *Hin*dIII/*Eco*RI marker.

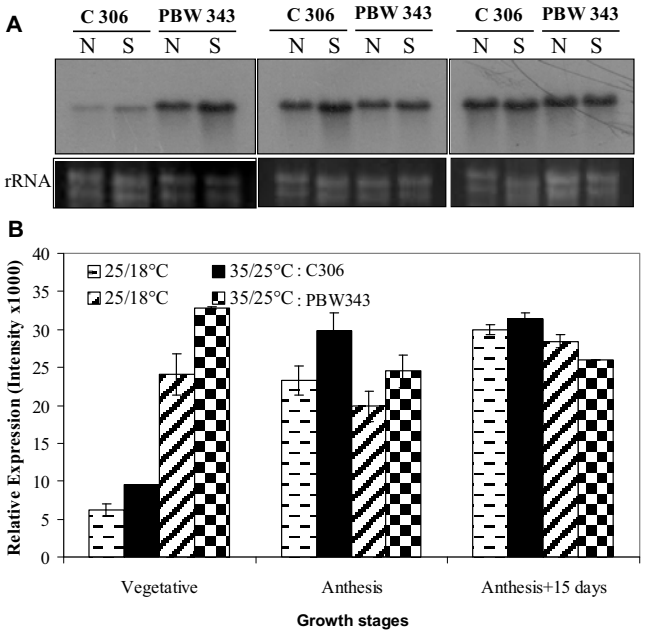

Fig. 6 Expression of *psbA* gene encoding 32kDa protein (D1) of photosystem II in response to high temperature stress. N, 25/18°C day/night; S, 35/25°C day/night; Plants were exposed to high temperature stress from 45 days after sowing till maturity. Leaf samples from control and high temperature treated plant were collected at the vegetative stage (7 days after HT), anthesis (30 days after HT) and 15 days after anthesis (45 days after HT). (**A**) Northern blot analysis of *psbA* gene; (**B**) Densitometric quantification of relative expression levels of *psbA*.

vity of Rubisco synthesis (transcription and translation) and Rubisco activase of these wheat varieties to long periods of heat stress. This could be the reason for photosynthetic reduction under elevated temperature and this in agreement with Sage and Kubien (2007) who reported that photosynthetic limitation above thermal optimum are heat labiality of Rubisco activase on one hand and a limitation in electron transport on the other. Also, Ristic *et al*. (2009) reported that Rubisco activase constrain the photosynthetic potential of plants at HT while Stasik and Jones (2007) suggested that difference in tolerance to heat stress in wheat related to the stability of Rubisco function rather than to the effect on PSII activity. Heat stress as well as other stresses can trigger some mechanisms of defense such as enhancing or suppression some genes or new genes that was not expressed under normal conditions (Feder 2006; Al-Whaibi 2011). Also Efeoglu and Terzioglu (2009) reported that HT stress induces drastic changes in gene expression in a wide variety of prokaryotic and eukaryotic organisms. To understand further, expression of genes encoding Rubisco large subunit (*rbcL*) and small subunit (*rbcS*) was examined by using RT-PCR. After 7 days of stress, 'C306' showed a reduction in the expression level of *rbcL*, while no reduction in 'PBW343' was observed. However, in the flag leaf, prolonged heat stress mediated reduction in the expression of *rbcL* was higher in 'PBW343' as compared with 'C306', indicating the susceptibility of chloroplast genome encoded *rbcL* gene expression in 'PBW343' (**Fig. 4**). Significant reduction in the expression of the nuclear encoded *rbcS* gene was observed in 'PBW343' in all stages, while in 'C306' the reduction in rbcS expression was considerable only after 45 days of HT (15 days after anthesis) as compared with their respective controls (**Fig. 5**). Thus, higher reduction in Rubisco activity under heat stress in 'PBW343' is, at least in part, due to the hypersensitivity of expression of *rbcS* in 'PBW343' under long-term heat stress.

In green plants, photosynthesis is among the first processes being affected by elevated temperature. The damage due to heat stress includes a wide range of structural and functional changes (Almeselmani *et al*. 2009b). The results from *in vivo* and *in vitro* studies showed that the primary target of HT stress involves the PSII reaction center with reduced water oxidizing activity being the major injury (Yordanov 1995). To further elucidate, whether sensitivity in expression of genes encoding proteins involved in photochemical reactions of photosynthesis is responsible for differential sensitivity of photosynthesis in 'C306' and 'PBW343', the expression of chloroplast gene *psbA* (32 kDa D1 protein of PSII) and nuclear gene *psbO* (oxygen evolving complex 33 kDa protein) were analyzed under prolonged heat stress. Northern blot analysis showed an increase in expression of *psbA* at vegetative stage and anthesis in response to heat stress in both the varieties, while after 45 days of HT, *psbA* levels were similar in both control and HT (**Fig. 6**). Expression of *psbO* was higher under heat stress at vegetative stage (7 days of HT), but later the expression levels under HT were comparable to their respective controls except that for 'PBW343' at 45 days of HT (**Fig. 7**). These results showed that the higher stability of photosynthesis in 'C306' under prolonged heat stress is mainly due to better tolerance in chl content and *chl a/b* ratio, and expression of Rubisco genes.

Significant increase in respiration rate was observed in both the genotypes during first week of heat stress as compared with respective control plants in both genotypes. However, when the plants were exposed to 30 days of heat stress (anthesis stage) and 45 days of heat stress (15 days after anthesis), the rate of leaf respiration was only marginally higher in 'C306', while 'PBW343' showed significantly higher respiration rate as compared with their respective control and 'C306' plants (**Fig. 8**). The phenomenon of plants to maintain similar respiratory rates when grown at different temperatures is referred to as respiratory homeostasis (Kurimoto *et al*. 2004a). The degree of homeostasis varies between 0 (no acclimation) and 1 (full acclimation). In wheat and rice it has been shown that high respiratory homeostasis is associated with high efficiency of respiratory

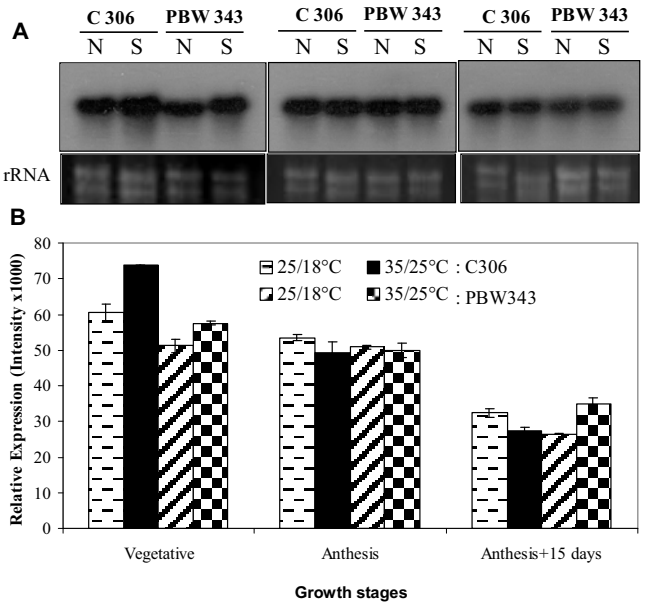

Fig. 7 Expression of *psbO* gene encoding a 33 kDa protein of oxygen evolving complex of photosystem II in response to high temperature stress. N, 25/18°C day/night; S, 35/25°C day/night; Plants were exposed to high temperature stress from 45 days after sowing till maturity. Leaf samples from control and high temperature treated plant were collected at the vegetative stage (7 days after HT), anthesis (30 days after HT) and 15 days after anthesis (45 days after HT). (**A**) Northern blot analysis of *psbO* gene; (**B**) Densitometric quantification of relative expression levels of *psbO*.

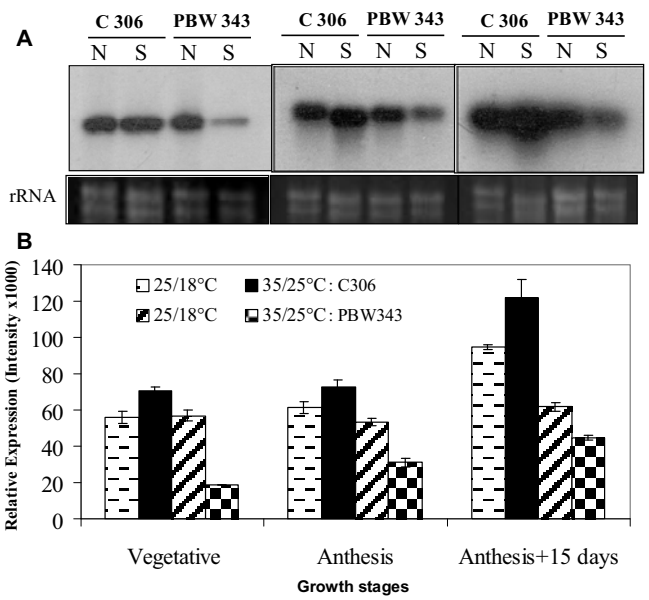

Fig. 9 *HSP101* expression in response to high temperature stress. N, 25/18°C day/night; S, 35/25°C day/night; Plants were exposed to high temperature stress from 45 days after sowing till maturity. Leaf samples from control and high temperature treated plant were collected at the vegetative stage (7 days after HT), anthesis (30 days after HT) and 15 days after anthesis (45 days after HT). (**A**) Northern blot analysis of *HSP101* gene; (**B**) Densitometric quantification of relative expression levels of *HSP101*.

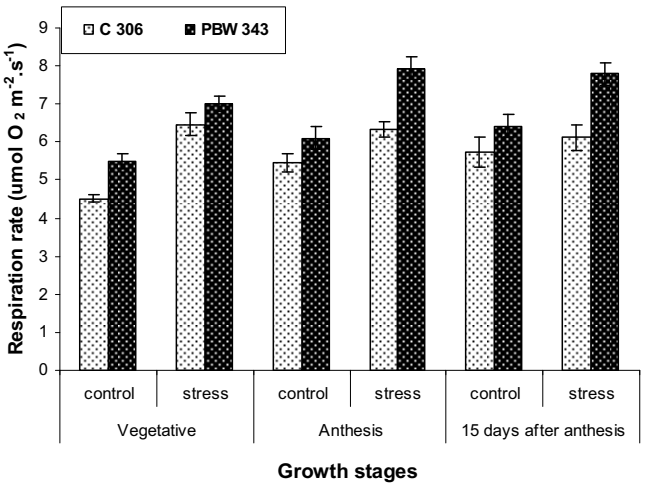

Fig. 8 Respiration rate (µmol O_2 m^{-2} s^{-1}) in C306 and PBW 343 under normal temperature (25/18°C) and high temperature (35/25°C) at vegetative stage, anthesis and 15 days after anthesis. Vertical bars indicate SE, n = 3; For genotype × temperature interaction CD at $P < 0.05$ = 3.17.

ATP production under chilling stress (Kurimoto *et al*. 2004b) and thus genotypes with high respiratory homeostasis maintained growth at low temperature (Kurimoto *et al*. 2004a). In this study, the respiration in flag leaf (vegetative stage and 15 days after anthesis) of heat tolerant 'C306' did not increase significantly (CD = 1.43, $P < 0.05$) under heat stress. However, in heat susceptible 'PBW343', the rate of respiration in the flag leaf was significantly higher under heat stress as compared with control (**Fig. 8**). This suggests that 'C306' has higher respiratory homeostasis than 'PBW343', when exposed to long periods of heat stress. While reviewing the response of plant respiration to temperature, Atkin *et al*. (2005) suggested that the assumptions of global carbon circulation models such as that plant respiration increases exponentially with temperature (with a constant Q_{10}) and there is no acclimation of respiration to long-term changes in temperature may be untenable. Results from this study suggest that long periods of HT stress lead to acclimation and genotypic variation is available in wheat.

Campbell *et al*. (2001) shown that HSP101 in wheat is induced by heat, drought and ABA. Expression of *HSP101* is heat-inducible in leaves of *Arabidopsis* (Schirmer *et al*. 1994), soybean (Lee *et al*. 1994), rice (Agarwal *et al*. 2003) and maize (Nieto-Sotelo *et al*. 2002). In wheat, HSP101 protein was detected in leaves until 7 days after germination, while in maize it was detected until 12 days after germination under normal growth temperatures (Young *et al*. 2001). Northern analysis showed that maize *HSP101* express constitutively in leaf no. 10 to leaf no. 17, and heat stress increased *HSP101* expression five fold in these leaves (Young *et al*. 2001). In leaves of lima bean, the HSP100/ClpB protein gene constitutively expressed, but transcript levels increased under heat stress (Keeler *et al*. 2000). It has been reported that there is genetic variability in the synthesis of HSPs in wheat (Ahn 2004; Efeoglu 2009). Consistent with these observations, Northern analysis revealed that *HSP101* is expressed constitutively in both the wheat genotypes examined in this study (**Fig. 9**). In heat tolerant genotype 'C306', expression level of *HSP101* showed up regulation at all stages and duration of HT. In contrast, heat susceptible genotype 'PBW343' showed considerable reduction in *HSP101* transcripts at all three durations of heat stress as compared with their respective controls (**Fig. 9**). *HSP101* induction was found to be transient in nature in rice (Agarwal *et al*. 2003) and lima bean (Keeler *et al*. 2000). In our study, heat-tolerant 'C306' showed up regulation of *HSP101* expression under long durations of heat stress. In contrast, heat-susceptible 'PBW343' did not show *HSP101* up regulation under prolonged HT (**Fig. 9**). These results suggest that *HSP101* expression is constitutive but heat stress-inducible in wheat leaves. Heat stress-induced up regulation of *HSP101* was maintained during prolonged heat stress only in heat tolerant genotype 'C306' but not in heat sensitive 'PBW343' (**Fig. 9**). Often induction of HSP expression requires raise in temperature over a short period. Why the expression of HSP101 is not up regulated/sustained in heat susceptible 'PBW343' under prolonged HT needs further study.

Genetic and transgenic analyses have shown that HSP101 is essential for acquired as well as basal thermotolerance (Queitsch *et al*. 2000; Hong and Vierling 2001; Nieto-Sotelo *et al*. 2002; Agarwal *et al*. 2003; Katiyar-Agarwal *et al*. 2003). Analysis of HSP101 function in yeast translation mutants suggested that the HSP101 is required for translational enhancement of capped mRNAs (Wells *et al*. 1998). HSP104/HSP101 mediates the resolubilization of heat-inactivated proteins from insoluble aggregates (Parsell *et al*. 1994; Agarwal *et al*. 2003). It has been suggested that HSPs general role is to act as molecular chaperones regulating the folding and accumulation of proteins as well as localization and degradation in all plants and animal species (Panaretou and Zhai 2008; Hu *et al*. 2009; Gupta *et al*. 2010). Since HSP101 is essential for thermotolerance, heat tolerance of 'C306' is at least in part due to sustained up regulation of *HSP101* expression under prolonged heat stress. Tripp *et al*. (2009) suggested that these proteins as chaperones prevent the irreversible aggregation of other proteins and participate in refolding proteins during heat stress conditions.

In conclusion, heat-tolerant 'C306' showed less reduction in photosynthesis and maintained respiration, while heat susceptible 'PBW343' showed higher reduction in photosynthesis accompanied by significant increase in respiration. Thus less photosynthesis and high respiration may lead to less biomass accumulation and productivity in heat susceptible genotypes. Better tolerance in photosynthesis to long-term heat stress in 'C306' can be attributed to less reduction in total chl and chl *b* content, Rubisco activity. Maintenance of Rubisco activity in 'C306' under HT may be due to maintenance of expression of *rbcS* and *rbcL* genes. Maintenance of high expression levels of *HSP101* during long-term heat stress in 'C306' may also be reason for better tolerance of 'C306' as compared with 'PBW343', as HSP101 plays a crucial role in repair of heat damaged proteins.

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