DOI: 10.1007/s11099-018-0808-6 PHOTOSYNTHETICA 56 (4): 1123-1133, 2018

Comparative chloroplast proteome analysis of exogenously supplied trehalose to wheat seedlings under heat stress

Y. LUO+, H.Y. LIU, Y.Z. FAN, W. WANG, and Y.Y. ZHAO

Instruments Sharing Platform of School of Life Sciences, East China Normal University, Shanghai 200241, China

Abstract

The aim of our study was to investigate the underlying molecular mechanisms of exogenously supplied trehalose affecting wheat photosynthesis under heat stress. The amount of ATP synthase (ATPase), oxygen-evolving enhancer protein (OEE), PsbP, Rubisco, chloroplast fructose-bisphosphate aldolase (FBPA), and ferredoxin-NADP(H) oxidoreductase (FNR) were downregulated, while PSI reaction center subunits were upregulated under heat stress. However, in the trehalose-pretreated groups, the amount of FNR, cytochrome b_6f complex, PSI reaction center subunits, ATPase, FBPA, and Rubisco were upregulated under normal growth conditions and heat stress. Besides, during the recovery period, the upregulation in CAB, PsbP, OEE2, and ATPase suggested that trehalose pretreatment might help to the recovery of PSII and PSI. These results indicate that trehalose pretreatment effectively regulates the levels of the photosynthesis-related proteins and relieves the damage of heat stress to wheat chloroplast.

Additional key words: chlorophyll fluorescence; chloroplast; photosynthesis-related proteome; PSI activity.

Introduction

The ambient temperature has increased steadily during this century (IPCC 2008). This continued warming trend may increase the frequency and severity of heat stress which can affect wheat photosynthesis, respiration, and uptake of mineral elements *etc*., thereby restricting the wheat growth and affecting the crop yield and quality of wheat. Wheat photosynthesis is very sensitive to high temperature (Wahid *et al.* 2007), which can reduce photosynthesis by destroying the structure and function of chloroplast and decreasing the chlorophyll (Chl) content (Qiang *et al.* 1995), and thus accelerate leaf senescence, and finally limit the growth and yield of wheat (Wang *et al*. 2011). Heat disasters often happen in the north of China and the Yangtze River region and reduce wheat production by 10–20% (Jin 1983). Therefore increasing the heat tolerance of wheat is very important during the wheat cultivation in the present situation.

Trehalose, widely distributed in nature, is a non-reducing disaccharide composed of two glucose units linked at their 1-C (Richards *et al.* 2002). Due to the characteristics of its chemical composition, trehalose can protect cellular components, including proteins and cell membrane under stress conditions. Therefore, trehalose is a kind of stress protectant. Wheat (El-Bashiti *et al.* 2005, Luo *et al.* 2010), rice (Garg *et al.* 2002), and yeast (*Baudoinia compniacensis*) (Al-Naama *et al.* 2009) can accumulate trehalose to protect their cells from the further damages and maintain themselves under normal conditions. Genetic approaches show that the expression of trehalose-biosynthetic genes (*TPS* and/or *TPP* genes) from yeast and *E. coli* in tomato and some other model plants increases their abiotic stress tolerance and improves photosynthesis (Lyu *et al.* 2013). However, in most cases, trehalose accumulation is too low to serve as an

Received 31 January 2017, *accepted* 28 July 2017, *published as online-first* 12 April 2018.
+Corresponding author, e-mail: 108lyin@163.com
Abbreviations: CAB – chlorophyll *a-b* binding protein; CEF – cyclic electron flow; Chl – chlorophyll; Cyt b_6f – cytochrome b_6f complex; $ETR_{(I)}$ – electron transfer rate of PSI; $ETR_{(II)}$ – electron transfer rate of PSII; F_0 – minimal fluorescence yield of the dark-adapted state; F_m – maximum fluorescence; F_v/F_m – the maximum quantum yield of PSII photochemistry; FAD – flavin adenine dinucleotide; FBPA – chloroplast fructose-bisphosphate aldolase; FM – fresh mass; FNR – ferredoxin-NADP(H) oxidoreductase; IEF – isoelectric focusing; MS – mass spectrometry; OEC – oxygen-evolving complex; OEE – oxygen-evolving enhancer protein; P_m – maximal P700 signal; PsaD – PSI reaction center subunit II; PsaE – PSI reaction center subunit IV; q_P – photochemical quenching coefficient; SD – standard deviation; TIL – temperature stress-induced lipocalin; $Y_{(I)}$ – photochemical quantum yield of PSI; $Y_{(II)}$ – effective photochemical quantum yield of PSII; $Y_{(NA)}$ – nonphotochemical PSI quantum yield of acceptor-side; $Y_{(ND)}$ – nonphotochemical PSI quantum yield of donor-side; $Y_{(NO)}$ – PSII quantum yield of nonregulated energy dissipation; $Y_{(NPQ)}$ – PSII quantum yield of regulated energy dissipation.
Acknowledgements: This work was financially supported by the Natural Science Foundation of China (No. 31200301).

osmoprotectant molecule (Wingler and Paul 2013). For example, the trehalose content detected in rice and tobacco is only about 10 μg g^{-1}(fresh mass, FM) and it also varies in different species. In our previous study, the winter wheat synthesized 502 μg(trehalose) g^{-1}(FM) under normal growth conditions, which increased to 1,250 μg g^{-1}(FM) under heat stress and to 1,658 μg g^{-1}(FM) in trehalose-pretreated seedlings (Luo *et al.* 2010), indicating that exogenously supplied trehalose can effectively increase the trehalose content in wheat and act as a protectant.

Recently, with the development of proteomics, we can learn more about the complex molecular mechanisms of plant responses to heat stress at the protein level. Except wheat, many crops, such as rice, barley, and soybean, have been studied in proteomics. Chloroplast, the organelle of photosynthesis, also possesses genetic material. However, its genome only encodes about 80–100 different kinds of proteins and the other about 2,500–3,500 different kinds of proteins are encoded by the nuclear genome (Martin and Herrmann 1998). Meanwhile, the functions of many proteins in chloroplast have not been thoroughly studied. Moreover, there is no detailed analysis about the influence of trehalose on wheat chloroplast proteome under heat stress. Our previous work showed that exogenously supplied trehalose protected chloroplast thylakoid membrane structure in a concentration-dependent manner (Luo *et al.* 2010). Therefore, we speculate that, under heat stress, exogenously supplied trehalose may also have influence on chloroplast proteome. In this paper, 1.5 mM trehalose is exogenously supplied to wheat seedlings and their physiologically relevant conditions are mimicked. We aimed to apply 2-DE technique to detect changes in the level of protein abundance and then identify the main proteins differentially expressed under heat stress. Meanwhile, we were looking forward to investigate the underlying molecular mechanism in response to exogenously supplied trehalose under heat stress in the wheat chloroplast.

Materials and methods

Plant growth and treatments: Seedlings of wheat (*Triticum aestivum* L. cv. Ning 13) grew hydroponically in Hoagland solution as described in Luo *et al.* (2008), in a 13-h day [PAR of 300 μmol(photon) m^{-2} s^{-1}] and 11-h night regime. When the second leaves were fully expanded, wheat seedlings were treated with Hoagland solution containing 1.5 mM trehalose for 3 d (Tre) or with Hoagland solution alone as a control (CK). After that, the seedlings were subjected to heat stress (40°C for 24 h), and then were kept at room temperature for another 12 h (for 2-D) and 24 h (for the determination of Chl fluorescence and P700) which were marked as R12 or R24, respectively.

Chl fluorescence and P700 measurements were carried out simultaneously using *Dual-PAM-100* fluorometer (*Walz*, Effeltrich, Germany) and the parameters were assessed using the induction curve recording mode in the *DUAL-PAM* software. After being for 30 min adapted in the dark, the minimal fluorescence yield (F_0) was immediately established. Then the maximum fluorescence (F_m) was detected using saturation pulse [10,000 μmol (photon) m^{-2} s^{-1}, 600-ms duration] and maximal quantum yield of PSII (F_v/F_m) was calculated as $(F_m - F_0)/F_m$ (Kramer *et al.* 2004; Schreiber 2004). A 620-nm light-emitting diode (LED) light and a PAR of 531 μmol(photon) m^{-2} s^{-1} from 460-nm LED blue actinic light were delivered to the wheat seedlings. The effective photochemical quantum yield of PSII [$Y_{(II)}$] and photochemical quenching (q_P) were automatically calculated by the *DUAL-PAM* software. After far-red preillumination, the maximal P700 signal (P_m) was detected by applying a single pulse. P_m' is analogous to the Chl fluorescence parameter F_m'. The nonphotochemical PSI quantum yield of acceptor side-limited heat dissipation [$Y_{(NA)}$] was calculated as $Y_{(NA)} = (P_m - P_m')/P_m$ (Pfündel 2008). The donor side-limited heat dissipation [$Y_{(ND)}$], the photochemical quantum yield of PSI [$Y_{(I)}$] and the electron transfer rate of PSI [$ETR_{(I)}$] were also automatically calculated by the software. All the measurements were repeated for at least 5 times.

Isolation and purification of chloroplasts: The collected leaves of wheat seedlings (the second and third leaves) were used to extract chloroplasts. The isolation protocols of chloroplasts from wheat leaves were performed according to Wang *et al*. (2013) with some modifications. All steps were performed in the dark at 4°C. Frozen wheat leaves were cut into small pieces with scissors and grounded to fine powder with mortar and pestle in liquid nitrogen. Then, isolation buffer I, containing 0.33 M sorbitol, 1 mM $MgCl_2$, 50 mM HEPES/KOH (pH 7.8), 2 mM EDTA, 10 mM DTT, 0.1% BSA, 5 mM sodium ascorbate, and 0.1% polyvinylpyrrolidone (PVP), was added. The homogenate was filtered through four layers of gauze. Then the filtrate was centrifuged at 2,000 × *g* for 10 min to harvest the crude chloroplasts. Next, the pellets were carefully resuspended in 5 mL of the isolation buffer II, containing 0.3 M sorbitol, 1 mM $MgCl_2$, 50 mM HEPES/KOH (pH 7.8), and 2 mM EDTA. Subsequently, the resuspended chloroplasts were loaded onto a *Percoll* step gradient buffer, which was combined with 5 mL of 40% *Percoll* and 4 mL of 80% *Percoll* in the isolation buffer II. Chloroplast separation was achieved by centrifugation at 16,000 × *g* for 20 min at 4°C. The green interphase between 40 and 80% *Percoll* was the purified intact chloroplasts. Then, the isolation buffer II was added to the chloroplasts and washed twice. The pellets were used immediately following the protein extraction protocols or stored at –80°C.

Extraction of chloroplast proteins was carried out as described by Phee *et al.* (2004) with some modifications. The chloroplast preparations were lysed in 1 mL of solubilization buffer (50 mM HEPES/KOH, pH 7.9, 4% CHAPS, 1 mM PMSF) and incubated on ice for at least 2 h and then centrifuged at 12,000 × *g* for 15 min at 4°C. Four volumes of 100% acetone were added into the supernatant, and it was stored at –20°C to –25°C for at least 2 h. Chloroplast proteins were collected by centrifugation at 15,000 × *g* for 15 min. Pellets were washed with precold acetone (–20°C ~ –25°C) for 3 times. Finally, the washed pellets were air-dried and ready for use or stored at –80°C.

2-D gel electrophoresis and isoelectric focusing: All the 2-D procedures were performed according to the manual of *Bio-Rad*. Protein samples were resuspended in a lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 0.2% *Bio-Lyte*) at room temperature for 30 min in order to achieve full denaturation, and then centrifugated at 20,000 × *g* for 15 min at room temperature. The supernatant contained the fully denatured proteins. Protein quantification was carried out using the Bradford protein assay kit. After quantification, a trace of bromophenol blue was added and mixed. For isoelectric focusing (IEF), immobilized gel strips (pH 4 – 7L, 17 cm; *Bio-Rad*, USA) were used to separate the protein lysate (the IEF temperature was set at 20°C). The procedure was modified for full and steady-state focusing. Briefly, the gel strip was rehydrated for 12 h at 50 V and the proteins were separated using the following stepwise increases in voltage and running times: 250 V for 30 min; 1,000 V for 1 h; 10,000 V for 5 h; and then 10,000 V (to a total for 60 kVh). After IEF, strip gels were incubated for 15 min at room temperature with equilibration buffer I (0.375 M Tris-HCl, pH 8.8, 6 M urea, 20% glycerol, 2% SDS, 1% DTT) and then transferred to equilibration buffer II (0.375 M Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 2.5% iodoacetamide) for another 15 min. After this equilibration step, the strip gels were placed on 12.5% denaturing acrylamide gels and sealed with a 0.5% agarose solution. SDS-PAGE was performed at 150 V for 8 h with a *PROTEAN II xl 2-D Cell* (*Bio-Rad*, USA). Protein spots in the 2-D gels were visualized by *Coomassie Brilliant Blue* (CBB G-250) staining.

Image analysis and protein identification: To analyze the expressed protein patterns, stained gels were scanned using a *Powerlook III* flatbed scanner (*UMAX*, Taiwan), followed by analysis of protein spots using *PDQuest v 8.0* (*Bio-Rad*, USA). The differentially expressed proteins were chosen by the spot density between different groups. The target protein spots were excised from the stained gels and transferred to *Sangon Corporation* (Shanghai, China) for Maldi-TOF/TOF-MS (*Applied Biosystems*, Foster City, USA). In gel digestion was performed according to Katayama *et al*. (2001). Samples were resuspended with 5 μL of 0.1% TFA followed by mixing in 1:1 ratio with a matrix consisting of a saturated solution of α-cyano-4-hydroxy-trans-cinnamic acid in 50% acetonitrile (ACN), 0.1% TFA, and then peptide mass spectrometry (MS) and MS/MS analyses were performed. Based on combined MS and MS/MS spectra, proteins were successfully identified according to 95% or higher confidence interval of their scores in the *MASCOT V2.3* search engine (*Matrix Science Ltd*., London, U.K.) against the *NCBInr-Viridiplantae* (green plants) database. The digestion enzyme was trypsin, and carbamidomethyl was considered as the fixed modification, while acetyl (protein N-term), deamidated, dioxidation, and oxidation were considered as partial modifications. One single missed cleavage, 100 ppm for precursor ion tolerance and 0.3 Da for fragment ion tolerance was permitted.

Statistical analysis: All comparisons were analyzed by one-way analysis of variance (*ANOVA*), LSD and *Tukey*'s test of multiple comparison analysis for the independent samples ($p<0.05$). Values were expressed as mean ± SE from at least three independent experiments.

Results

Chl fluorescence and P700 measurement: Chl fluorescence is often used to measure the changes of PSII activity under stress conditions. In the CK groups, F_v/F_m declined under heat stress (Fig. 1*A*). Meanwhile, a similar pattern of other parameters including $Y_{(II)}$ (Fig. 1*B*), q_P (Fig. 1*C*), and electron transfer rate in PSII [$ETR_{(II)}$] (Fig. 1*D*) was observed under heat stress. However, in the Tre groups, these parameters were significantly higher than those in the CK groups under heat stress and during heat recovery period, and maintained at the similar levels to the nonstressed seedlings (Fig. 1). These results indicated that exogenously supplied trehalose could relief the damage of heat stress to the photosynthetic apparatus, and thus diminish its adverse effect on the photochemical efficiency. Meanwhile, heat stress induced the increase in $Y_{(NO)}$ and the decrease in $Y_{(NPQ)}$ (Fig. 1*E*, *F*), and the $Y_{(NO)}$ in the Tre group was lower than that in CK group (Fig. 1*E*).

P700 parameters reflect a state of PSI. Heat stress decreased the photochemical quantum yield of PSI [$Y_{(I)}$] (Fig. 2*D*) and $ETR_{(I)}$ (Fig. 2*E*) compared with CK groups. But the maximal P700 signal (P_m) (Fig. 2*A*) showed no significant changes during heat stress, which indicated that the photosynthetic activity of PSI may be generally stable under heat stress and during the subsequent recovery period. Meanwhile, Tre pretreatment made no significant effects on P700 parameters (Fig. 2) under normal condition, but $Y_{(ND)}$ (Fig. 2*B*) significantly increased under heat stress, indicating that the light protection mechanism for PSI in

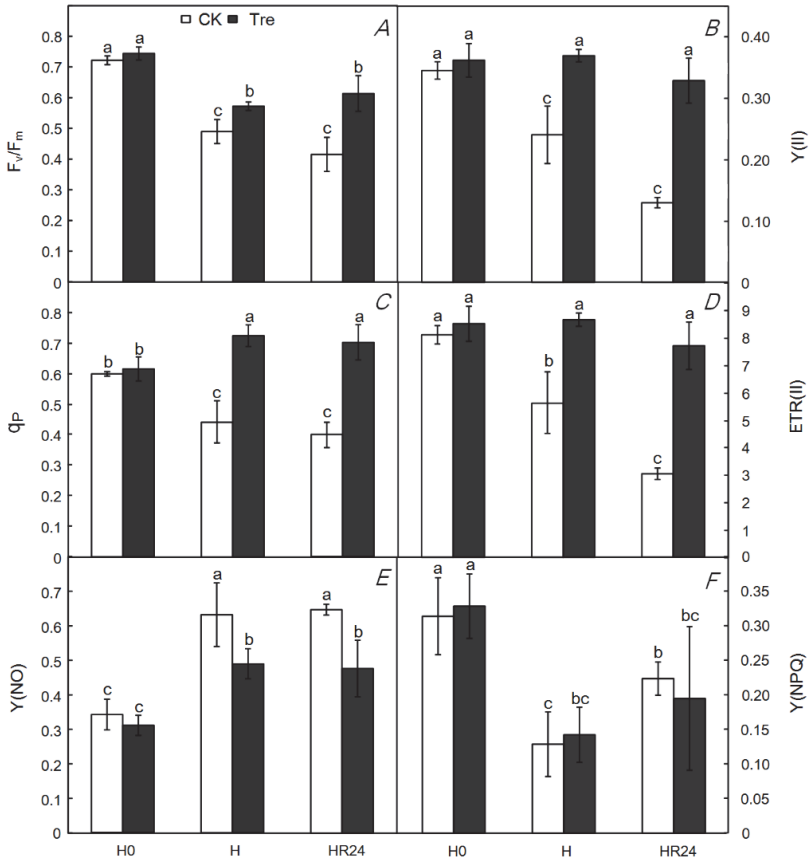

Fig. 1. Influence of exogenous trehalose on chlorophyll fluorescence parameters: F_v/F_m (*A*), Y(II) (*B*), q_P (*C*), ETR(II) (*D*), Y(NO) (*E*), and Y(NPQ) (*F*) of wheat leaves under different treatments. CK – control group; Tre – trehalose pretreatment for 3 d; H0 – without high temperature treatment; H – high temperature treatment for 24 h; HR24 – recovered 24 h from high temperature treatment. *Different letters* show significant differences (P<0.05).

$Y_{(ND)}$ enhanced (Fig. 2*B*), but the $Y_{(I)}$ (Fig. 2*D*) was lowered at the same time, indicating that the light protection mechanism for PSI was promoted to a certain extent.

Wheat chloroplast proteome: Through image analysis with *PDQuest* software, more than 100 highly reproducible chloroplast protein spots were identified on each gel (Fig. 3). From these spots based on three replications, 19 spots were revealed as differentially expressed proteins with pI values ranging from 4 to 7 and with relative molecular masses of 10 to 200 kDa.

Compared with CK plants, Tre pretreatment upregulated the amount of cytochrome b_6f complex (Cyt b_6f) iron-sulfur subunit, ferredoxin-NADP(H) oxidoreductase (FNR), and PSI reaction center subunit II (PsaD), ATP synthase (ATPase) subunit gamma in the photosynthetic electron transport chain. Besides, the chloroplast fructose-bisphosphate aldolase (FBPA), Rubisco large subunit in the carbon reaction were also upregulated (Fig. 3*A*). However, the contents of PSI reaction center subunit IV (PsaE), PsbP, OEE2, and the predicted chloroplastic-like protein TIC110 were downregulated (Fig. 3*A*, Table 1).

Under heat stress, Tre pretreatment significantly increased the amount of ATPase CF1 beta subunit and FBPA (Fig. 3*B*, Table 1). Meanwhile, the contents of FNR were also upregulated (Fig. 3*B*, Table 1). In contrast, the amount of the proteins including Cyt b_6f iron-sulfur subunit 2, Chl *a*–*b* binding protein (CAB), Rubisco large subunit, chloroplastic ATPase delta chain, PsbP, OEE2, and the hypothetical protein TRIUR3_05353 were downregulated in wheat (Fig. 3*B*, Table 1).

After recovery from heat stress, compared with the CK (without Tre pretreatment), Tre pretreatment upregulated the levels of CAB, FBPA, PsbP, OEE2, ATPase delta chain, and CF1 alpha subunit, while the amount of ATPase CF1 beta subunit, OEE1, temperature stress-induced lipocalin (TIL), Rubisco large subunit, and FNR were generally downregulated (Fig. 3*C*, Table 1). Besides, some hypothetical proteins, such as hypothetical protein TRIUR3_26766, the predicted chloroplastic-like protein TIC110, and plastid-lipid-associated protein 13, also showed lower contents in Tre groups than those in CK plants (Fig. 3*C*, Table 1).

Under heat stress, the amount of Cyt b_6f iron-sulfur subunit 2, CAB, PsaD, PsaE, the predicted chloroplastic-like protein TIC110, and plastid-lipid-associated protein 13 in wheat chloroplasts were upregulated (Fig. 3*D*, Table 1). However, the levels of OEE1 and OEE2, ATPase delta chain, ATPase CF1 alpha subunit, ATPase beta subunit, Rubisco large subunit, FBPA, FNR, and PsbP showed the downregulation in the heat-stressed groups (Fig. 3*D*, Table 1).

Discussion

Previously, we found that the proteins in the thylakoid membranes and photosynthetic capacity damaged by heat stress could be protected by Tre pretreatment in wheat seedlings (Luo *et al.* 2010). However, in this study, when exposed to heat stress, the level of CAB, which serves to maximize and regulate light harvesting (Silva *et al.* 2016), was upregulated (Fig. 3*D*, 3.19 ± 0.19). Silva *et al.* (2016) reported that the *PgCAB1* and *PgCAB2* expression levels significantly increased compared with control under NaCl stress, which suggested the specific function of PSII in the regulatory role in ginseng response to salinity and drought and this also might represent a strategy to prevent light stress-induced damage. We speculated that the plant itself might depress its energy supply to photosynthesis because of the heat stress and thus hope to meet the needs of energy and its metabolic balance by enhancing light harvesting in order to respond to this decrease in photosynthesis. Similarly, the lower content of CAB in the Tre-pretreated groups compared with CK group under heat stress (Fig. 3*B*, 0.46 ± 0.16) may be due to the reason that other response ways have compensated some of the needs. In accordance with the enhancement of Chl fluorescence parameters including the F_v/F_m, $Y_{(II)}$, and q_P (Fig. 1) in the Tre groups under heat stress, the level of CAB 8 in Tre groups was also generally upregulated (Fig. 3*C*, 2.01 ± 0.04) after recovery from heat stress, suggesting that during the subsequent recovery period, trehalose can promote plant metabolism by enhancing light harvesting, and therefore repair the damage caused by heat stress. The thermolability of PSII can depress photosynthetic electron transport in plants and may induce the ROS generation. And in the present work, we found that the $ETR_{(II)}$ significantly decreased under heat stress (Fig. 1*D*). When exposed to heat stress, oxygen-evolving complex (OEC) would separate from PSII (Hu *et al.* 2004) which is consistent with the downregulation of PsbP and OEE under heat stress (Fig. 3*D*, 0.62 ± 0.05; 0.62 ± 0.05). It was reported that PsbP, one of the extrinsic OEC proteins, is essential for the normal PSII function in higher plants *in vivo* (Ifuku *et al.* 2005), and it plays an important role in the dynamic life cycle of PSII (Ifuku *et al.* 2008, Ifuku *et al.* 2011). In this way, the upregulation of the PsbP content in the Tre-treated group during the recovery period helps to the recovery of PSII, which is in agreement with the result reported by Mamedov *et al.* (2015) that trehalose significantly stimulates the steady-state rate of O_2

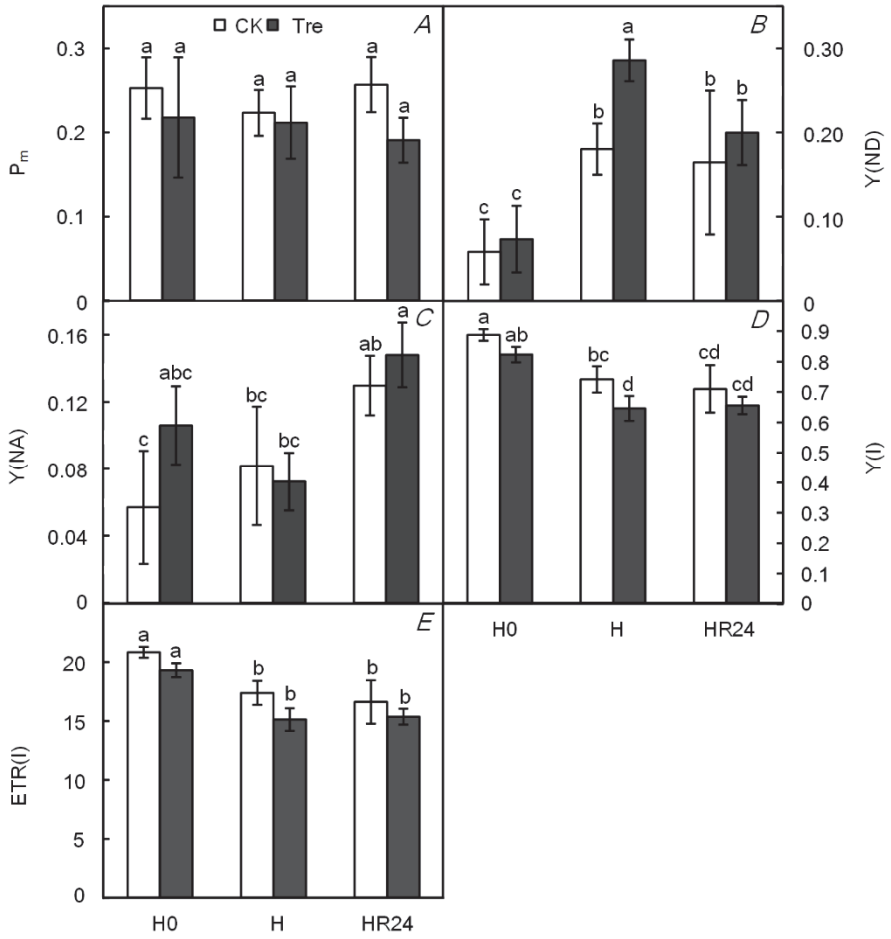

Fig. 2. Influence of exogenous trehalose on P700 parameters: P_m (*A*), Y(ND) (*B*), Y(NA) (*C*), Y(I) (*D*) and ETR(I) (*E*) of wheat leaves under different treatments. CK – control group; Tre – trehalose pretreatment for 3 d; H0 – without high temperature treatment; H – high temperature treatment for 24 h; HR24 – recovered 24 h from high temperature treatment. *Different letters* show significant differences (P<0.05).

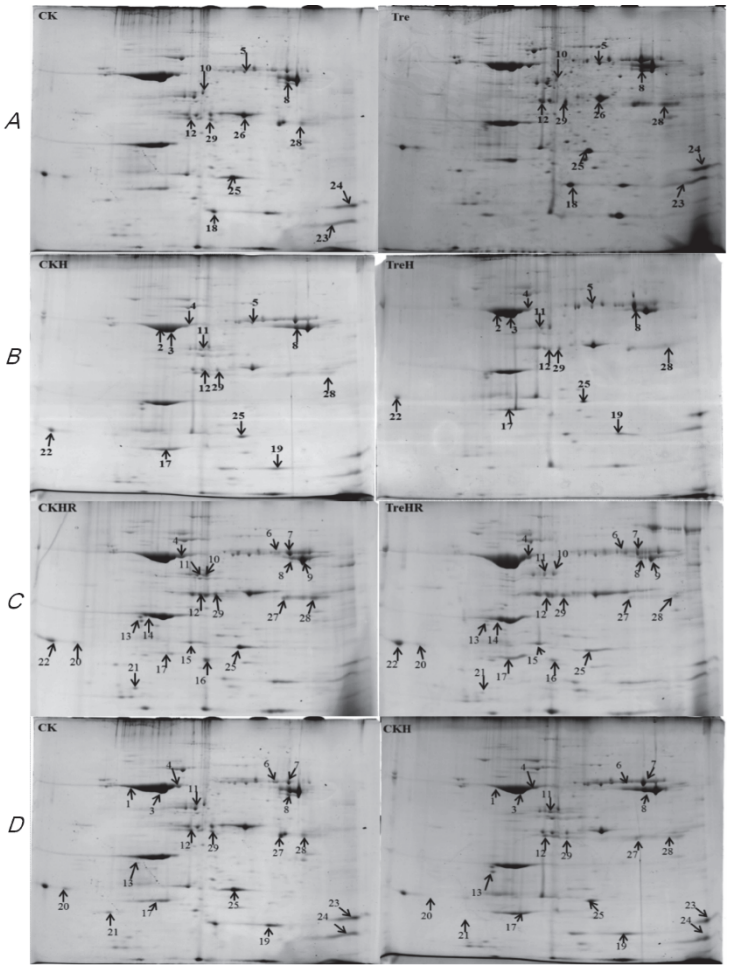

Fig. 3. Effects of exogenously supplied trehalose and heat stress on protein levels in wheat control seedlings (CK) and plants that pretreated with trehalose for 3 d (Tre). (*A*) gel from the plants without heat stress, (*B*) gel from the plants suffered from heat stress for 24 h, (*C*) gel from the plants recovered for 12 h, (*D*) gel from the plants before heat stress and after heat stress. H – high temperature treatment for 24 h; HR – recovered from high temperature treatment. Proteins were extracted from leaf chloroplasts, separated by 2-DE, and visualized by CBB G-250 staining. The numbers were the ID of these proteins.

evolution in both PSII membrane fragments and core complexes from spinach.

PsaD and PsaE are two subunits in PSI reaction center; PsaE is associated with cyclic electron flow (CEF) around PSI. They both were upregulated in wheat seedlings in the CK and Tre groups under heat stress (Fig. 3*B*,*D*, 3.15 ± 0.68, 2.46 ± 0.36). The upregulated PsaE under heat stress was in agreement with the result that heat stress significantly increased $Y_{(ND)}$ (Fig. 2*B*), suggesting that the light protection mechanisms of PSI in wheat seedlings improved under heat stress. Morgan-Kiss *et al.* (2002) suggested that suitable high temperature could improve the electron transfer ability mediated by PSI. And Hu *et al.* (2004) reported that the heat stress had no effect on the secondary structure of PSI when the temperature is below 50°C. PSI-CEF was confirmed to protect PSI against photodamage in heat-stressed leaves. So, the stable PSI activity may be due to the upregulated PSI subunits under heat stress. Our results were consistent with previous study by Zhang and Liu (2016) that heat stress (25–40°C) increased the PSI activity in *Arthrospira (Spirulina)*.

It was reported that when exposed to 42°C for 12 h, the content of FNR in soybean leaves was diminished (Ahsan *et al.* 2010). Heat stress-induced downregulation in the protein level of FNR (Fig. 3D, 0.42±0.10–0.55±0.09) slowed down the process of photosynthetic electron transport, while under the Tre-pretreatment conditions, the upregulation of FNR (Fig. 3*A*,*B*, 1.73 ± 0.04, 1.96 ± 0.17) helped protect wheat seedlings against the heat stress effects on the photosynthetic electron transfer process. Meanwhile, the amount of Cyt b_6f iron-sulfur subunit also increased in the Tre-pretreated groups (Fig. 3*A*, 1.59 ± 0.04). Previous studies showed that the formation of FNR–Cyt b_6f contributed to the CEF process (Zhang *et al.* 2001; Szymańska *et al.* 2011) and we also found that exogenously supplied Tre stimulated CEF (data not published). Therefore, it is possible that Tre pretreatment may contribute to heat tolerance in wheat seedlings through the CEF-induced protection mechanism.

The downregulation in the protein levels of ATPase CF1 α (Fig. 3*D*, 0.13 ± 0.01) and β subunit (Fig. 3*D*, 0.41 ± 0.14) and δ chain (Fig. 3*D*, 0) under heat stress indicated that heat stress can damage the energy metabolism process of wheat seedlings since the catalytic site for ATP synthesis is localized primarily on the β subunit. Similar to the results reported by Laino *et al.* (2010) in durum wheat seeds, our results showed that the amount of ATP-synthase decreased under heat stress. Moreover, Tre pretreatment

Table 1. General features of wheat chloroplast proteins in response to heat stress, as identified via MALDI-TOF/TOF-MS. Some proteins were not detected in some sample. gi – NCBI acc. No., TMM/pI – theoretical molecular mass/theoretical pI value, EMM/pI – experimentally determined molecular mass/pI value, PM – peptide matches, FC – fold-change, blank – not differentially regulated, "0" – not found, Tre – trehalose pretreatment, CK – control group, H – heat stress, R12 – recovered 12 h from heat stress treatment.

NO.	Accession No.	Protein description	TMM(kDa)/pI	EMM(kDa)/pI	PM	FC ± SD Tre/CK	TreH/CKH	TreHR12/CKHR12	CKH/CK
1	gi|14017579	ATP synthase CF1 beta subunit (chloroplast) [*Triticum aestivum*]	53.88/5.06	54/5.0	11				0.41 ± 0.14
2	gi|14017579	ATP synthase CF1 beta subunit (chloroplast) [*Triticum aestivum*]	53.88/5.06	54/5.0	11		9.80 ± 2.40		
3	gi|14017579	ATP synthase CF1 beta subunit (chloroplast) [*Triticum aestivum*]	53.88/5.06	54/5.0	11		1.71 ± 0.48		0.47 ± 0.13
4	gi|525291	ATP synthase beta subunit [*Triticum aestivum*]	59.33/5.56	55/5.3	8		2.29 ± 0.53	0.38 ± 0.04	2.23 ± 0.39
5	gi|81176509	atp1 (mitochondrion) [Triticum aestivum]	55.56/5.70	55/6.0	8	1.68 ± 0.05	0.57 ± 0.02		
6	gi|14017569	ATP synthase CF1 alpha subunit (chloroplast) [*Triticum aestivum*]	55.32/6.11	55/6.2	10			4.44 ± 0.16	0.13 ± 0.01
7	gi|14017569	ATP synthase CF1 alpha subunit (chloroplast) [*Triticum aestivum*]	55.32/6.11	55/6.2	10			0.54 ± 0.003	2.06 ± 0.13
8	gi|14017580	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast) [*Triticum aestivum*]	53.45/6.22	53/6.3	8	1.77 ± 0.05	0.45 ± 0.20	0.77 ± 0.004	0.21 ± 0.14
9	gi|51859663	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast) [*Psathyrostachys fragilis subsp. villosus*]	53.70/6.13	53/6.3	9			0.58 ± 0.08	
10	gi|721659748	PREDICTED: protein TIC110, chloroplastic-like [*Brachypodium distachyon*]	46.97/6.36	43/5.3	7	0.61 ± 0.01		0.54 ± 0.04	1.38 ± 0.01
11	gi|473755341	Hypothetical protein TRIUR3_05353 [*Triticum urartu*]	24.78/5.50	40/5.5	7		0.53 ± 0.02	0.69 ± 0.01	
12	gi|223018643	Chloroplast fructose-bisphosphate aldolase [*Triticum aestivum*]	42.22/5.94	37/5.7	6	0.64 ± 0.02	0.55 ± 0.09	0.59 ± 0.01	0.63 ± 0.06
13	gi|573942949	Probable plastid –lipid -associated protein 13, chloroplastic-like, partial [*Oryza brachyantha*]	31.11/5.52	30/5.0	2			0.48 ± 0.01	2.02 ± 0.32
14	gi|474352688	Oxygen-evolving enhancer protein 1, chloroplastic [*Triticum urartu*]	34.64/5.75	32/5.0	9			0.08 ± 0.004	

Table continues on the next page

Table 1 continued

NO.	Accession No.	Protein description	TMM(kDa)/pI	EMM(kDa)/pI	PM	FC ± SD Tre/CK	TreH/CKH	TreHR12/CKHR12	CKH/CK
15	gi|474121685	Chlorophyll *a-b* binding protein 8, chloroplastic [*Triticum urartu*]	29.30/8.69	22/5.4	9			2.01 ± 0.04	
16	gi|18650668	Temperature stress -induced lipocalin [Triticum aestivum]	21.81/5.50	19/5.5	9			0.71 ± 0.01	
17	gi|225690794	chlorophyll a-b binding protein [*Triticum aestivum*]	27.24/5.42	20/5.2	4		0.46 ± 0.16	2.38 ± 0.04	3.19 ± 0.19
18	gi|68566191	Cytochrome *b6f* complex iron-sulfur subunit, chloroplastic	24.11/8.47	17/5.7	9	1.59 ± 0.04			
19	gi|586663462	PREDICTED: cytochrome *b6f* complex iron-sulfur subunit 2, chloroplastic [*Amborella trichopoda*]	23.675/7.60	17/6.3	9		0.38 ± 0.07		3.17 ± 1.34
20	gi|475627717	ATP synthase delta chain, chloroplastic [*Aegilops tauschii*]	17.72/4.49	21/4.3	8			1.48 ± 0.05	0
21	gi|474352688	Oxygen-evolving enhancer protein 1, chloroplastic [*Triticum urartu*]	34.64/5.75	20/4.8	7			0.72 ± 0.03	0
22	gi|475627717	ATP synthase delta chain, chloroplastic [*Aegilops tauschii*]	17.72/4.49	22/4.1	8		0.46 ± 0.10	1.93 ± 0.04	
23	gi|131176	Photosystem I reaction center subunit IV; Short=PSI-E	15.45/9.82	15/7.0	4	0.23 ± 0.03			2.46 ± 0.36
24	gi|474150013	Photosystem I reaction center subunit II [*Triticum urartu*]	17.86/9.77	17/7.0	10	3.17 ± 0.63			3.15 ± 0.68
25	gi|474077556	Oxygen-evolving enhancer protein 2, chloroplastic [*Triticum urartu*]	25.71/8.94	23/5.9	9	0.54 ± 0.03	0.54 ± 0.11	1.64 ± 0.15	0.62 ± 0.05
	gi|767259516	Chain A, The Crystal Structure Of PsbP From Zea Mays	20.17/5.96	23/5.9	5				
26	gi|474125362	ATP synthase subunit gamma, chloroplastic [*Triticum urartu*]	52.62/7.52	38/6.0	11	1.62 ± 0.17			
27	gi|20302471	Ferredoxin-NADP(H)oxidoreductase [Triticum aestivum]	39.18/8.29	36/6.6	10			0.41 ± 0.02,	0.42 ± 0.10
28	gi|20302471	Ferredoxin-NADP(H)oxidoreductase [*Triticum aestivum*]				1.73 ± 0.04	1.96 ± 0.17	0.67 ± 0.05	0.55 ± 0.09
29	gi|223018643	Chloroplast fructose-bisphosphate aldolase [*Triticum aestivum*]	42.22/5.94	37/5.7	6	2.25 ± 0.10	1.83 ± 0.37	1.77 ± 0.03	0.43 ± 0.04

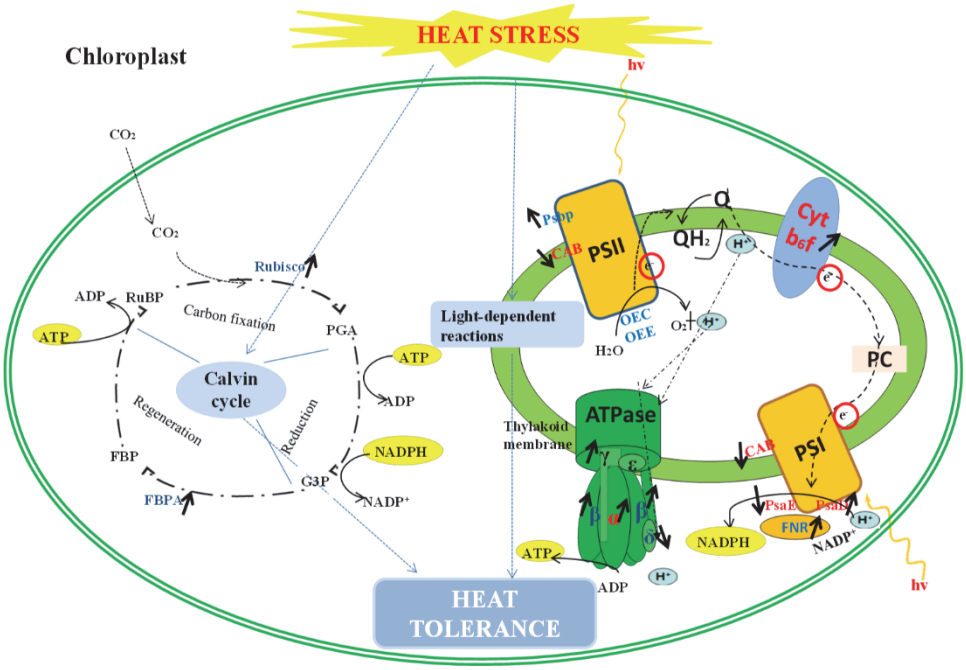

Fig. 4. Schematic presentation of a mechanism for heat tolerance in trehalose-pretreated wheat seedlings. This picture was redrawn from Wang *et al*. (2013) with some modification. Proteins under heat stress alone are integrated and indicated in red (upregulated) or blue (downregulated), respectively, while the protein level changes in trehalose pretreatment are indicated with *arrows* (up or down). ADP – adenosine diphosphate; ATP – adenosine triphosphate; ATPase – ATP synthase; CAB – chlorophyll *a*/*b* binding protein; Cyt b_6f – cytochrome b_6f; FBP – fructose-1,6-bisphosphate; FBPA – fructose-bisphosphate aldolase; FNR – ferredoxin-NADP(H) oxidoreductase; G3P – glyceraldehydes-3-phosphate; LHC – light-harvesting complex; NADP+/NADPH – nicotinamide adenine dinucleotide phosphate; OEC – oxygen-evolving complex; OEE – oxygen-evolving enhancer protein; PC – plastocyanin; PGA – 3-phosphoglycerate; Q – quinone; QH_2 – reduced quinone; RCP – reaction center protein; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP – ribulose-1,5-bisphosphate.

upregulated the amount of the ATPase γ subunit (Fig. 3*A*, 1.62 ± 0.17) before heat stress, while the amount of ATPase CF1 β subunit was upregulated in Tre-pretreated groups during heat stress (Fig. 3*B*, 9.80 ± 2.40), suggesting that Tre can reduce the heat stress-induced damage to energy metabolism. It had also been proposed that the CF1 α subunit displays an organizing function during the assembly of ATP synthase when exposed to the thermal denaturation (Wang *et al.* 1993). Therefore, the up-regulation of ATPase CF1 α subunit (Fig. 3*C*, 4.44 ± 0.16) in Tre-treatment group after recovery from heat stress possibly helped to re-establish the energy production system.

It is now well confirmed that increasing temperature inhibits photosynthetic CO_2 fixation in many plant species largely by the decrease in the activation state of Rubisco (Majoul *et al.* 2004). Therefore, the upregulation in the protein content of Rubisco large subunit caused by exogenously supplied Tre (Fig. 3*A*, 1.77 ± 0.05) might indicate that Tre pretreatment before heat stress helped strengthen photosynthesis efficiency. Moreover, the Tre pretreatment significantly enhanced the amount of FBPA in the chloroplast, and this would lead to the effective improvement in carbon assimilation process and therefore alleviated the inhibition by heat stress in plants.

The changes of proteins shown in Fig. 4 indicate that light-dependent reactions in Tre-pretreated seedlings were maintained by upregulating the component proteins including PsbP, Cyt b_6f, FNR, PsaD, and ATPase, while the normal function of the Calvin cycle was kept mainly by the upregulation of Rubisco and FBPA under heat stress. The upregulation of Cyt b_6f helped enhance the CEF around PSI which is involved in the maintenance of ATP production. Meanwhile, the upregulation of ATPase and FNR in Tre-pretreated group also helped maintain the production of ATP and NADPH under heat stress which can lead to the reduced generation of ROS. Furthermore, the contents of other proteins were also changed, for example, TIL and plastid-lipid-associated protein 13 (Table 1). This might be due to the damage to chloroplast membrane system caused by heat stress. It is reported that the basal level of TIL under normal conditions is relatively high (Chi *et al.* 2009), and we only found that the TIL was upregulated visually during the recovery period compared to the normal and heat-stress conditions. This result was consistent with the MDA content under heat stress and the recovery period in our previous work (the MDA content increased after heat stress in both CK and Tre groups, but Tre-pretreatment alleviated the MDA increase during the recovery period) since it was proposed that TIL could

protect plant against lipid peroxidation (Chi *et al.* 2009). The role of these proteins in heat tolerance is not known clearly. They may be directly related to heat stress response by an unknown mechanism or, indirectly, be a target for other proteins involved in the heat shock response (Laino *et al.* 2010). However, we did not detect the heat-shock protein in this experiment.

Conclusion: To the best of our knowledge, this current report is the first one to present changes in the chloroplast proteome of wheat seedlings pretreated with trehalose under heat stress. In conclusion, trehalose pretreatment caused upregulation of Cyt b_6f iron-sulfur subunit, PsaD, and FNR and it could contribute to the improvement of the CEF-related protection for PSI and PSII. Moreover, the enhancement in the protein contents of CAB, OEE, and Cyt b_6f iron-sulfur subunit in the trehalose-pretreated groups helped relieve the damage of heat stress to photochemical efficiency and the electron transfer rate. The upregulation of ATPase, Rubisco, and FBPA suggested that trehalose pretreatment could effectively ameliorate the heat stress-induced damage to the energy synthesis and carbon reaction processes.

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