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ORIGINAL PAPER

Glycine betaine application in grain filling wheat plants alleviates heat and high light-induced photoinhibition by enhancing the *psbA* transcription and stomatal conductance

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Abstract Wheat (*Triticum aestivum*) plants often suffer from heat stress combined with high irradiance during the grain filling stage. Glycine betaine (GB) is a compatible solute that accumulates rapidly to enhance stress tolerance in wheat under abiotic stress. In this study, the effect of foliar application of GB on photosystem II (PSII) photochemistry in wheat leaves under the heat and high light stresses were investigated. The results showed that GB-supplemented wheat plants maintained higher chlorophyll content, photochemical activity of PSII and net photosynthetic rate (P_n) than stressed only plants. Moreover, GB-treated plants could retard the decrease of *psbA* gene transcription and accelerate the endogenous accumulation of GB in leaves. The results suggested that the increasing stress tolerance by GB accumulation was associated with an improvement in D1 protein synthesis, which accelerated the repair of PSII following stress-enhanced photoinhibition. Moreover, the accumulation of abscisic acid under the heat and high light stresses was retarded by foliar application of GB, and the decrease of stomatal conductance was inhibited as well. The results suggested that GB accumulation in vivo was involved in the regulation of stomatal conductance. Further work is required to elucidate the mechanism of GB-induced stomatal movement and PSII photoprotection.

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Keywords Wheat (*Triticum aestivum* L.) · Glycine betaine · Photosystem II · *psbA* transcription · Stomatal conductance

Abbreviations

ABA	Abscisic acid
ANOVA	One-way analysis of variance
C_t	Cycle threshold
ddH_2O	Double distilled H_2O
ELISA	Enzyme-linked immuno sorbent
F_m	Maximal fluorescence
F_v	Variable fluorescence in dark-adapted leaves
F_v/F_m	Maximal efficiency of PSII
GB	Glycine betaine
g_s	Stomatal conductance
HPLC	High-performance liquid chromatography
P_n	Net photosynthetic rate
PPFD	Photosynthetic photon flux density
PSII	Photosystem II
ROS	Reactive oxygen species
SA	Salicylic acid

Introduction

Wheat (*Triticum aestivum* L.) is one of the most important food crops planted worldwide. Heat and high light stresses are the restriction factors that pose a major risk to food security. With the prospective global warming, the degree of wheat suffering from the heat and high light stresses is supposed to be more and more serious (Ortiz et al. 2008). Increasing occurrences of severe heat and high light events, especially during the grain filling stage, have become a

major problem for cereal production increasing the risk of significant yield losses and reductions in grain quality (Zhao et al. 2011).

In photosynthesis system of plants, reaction center in photosystem II (PSII) is the most susceptible component to many kinds of stresses such as heat, light or their combinations (Murata et al. 2007; Tyystjärvi 2013; Vass 2012). It has been proven that the extent of damage under stress depends mainly upon the balance between injury and repair of PSII (Baker 2008; Murata et al. 2007). Among the more than 25 kinds of subunits in PSII protein complex, the D1 protein encoded by the chloroplast *psbA* gene is one of the target site damaged by many environmental stresses (Yamamoto et al. 2008). Hence, fast turnover of D1 protein is considered as a premise for functional recovery of PSII (Yamamoto et al. 2008). Because the grain-filling stage is the reproductive phase, photosynthesis of wheat plants in this stage is extremely sensitive to environmental stress (Shah and Paulsen 2003). Therefore, much attention is currently being focused on the photoprotection mechanism of PSII in wheat plants suffering from heat stress combined with high irradiance during the grain-filling stage.

It is actually well established that glycine betaine (GB) plays an important role in mediating the main responses of plants to environmental stresses such as heat and strong light (Chen and Murata 2008). Excessive accumulation of GB in transgenic tomato showed an increased tolerance to heat-enhanced photoinhibition, which was associated with an improvement in D1 protein content and an acceleration in the repair of PSII. The GB alleviation was also connected closely with the enhancing antioxidative defense and alleviating lipid peroxidation (Li et al. 2014). An experiment upon transgenic cyanobacterium, *Synechococcus* sp. PCC 7,942 also showed that the accumulation of GB alleviated the inhibitory effect of moderate heat stress on the repair of PSII by accelerating the synthesis of the D1 protein (Allakhverdiev et al. 2007). Applied exogenously of GB or generated by transgenes for GB biosynthesis can enhance thermotolerance via the induction of the expression of certain stress-responsive genes, including those for enzymes that scavenge reactive oxygen species (ROS), the suppression of the accumulation of ROS, and also via the enhanced repair of PSII (Chen and Murata 2011). These results have enhanced our understanding of mechanisms that GB accumulation protects plants against stresses. However, few research was reported until now about the regulation of GB on the transcription of *psbA* gene in heat and high light stressed wheat plants, especially during the grain filling stage.

Another photoprotection mechanism of exogenous materials on higher plants under various environmental stresses is through the regulation of stomatal closure. The opening of stomata is essential for the supply of CO_2 for photosynthesis, but it is also harmful because plants PSII can be damaged, especially when plants were suffering from environmental stress (Schroeder et al. 2001). Others previous studies have shown that, excessive accumulation of GB increased the stomatal conductance in wheat seedlings under the heat stress (Wang et al. 2010b). However, little is known regarding the correlationship between the photoinhibition alleviation of GB application and stomatal movement in the grain filling wheat plants suffering from heat and high light stresses.

In this study, the photosynthetic response to heat and high light stresses of wheat during the grain filling stage were presented. Moreover, the result of supplementing a stressed wheat plant with foliar GB was also evaluated in order to understand a possible protective effect against photoinhibition induced by GB, especially through modulating the *psbA* gene transcription and stomatal movement.

Materials and methods

Plant materials and treatments

Wheat (*Triticum aestivum* L. cv. Aikang) seeds of uniform size were surface-sterilized with 1 % NaClO for 10 min, and grown in a plastic pot (height 25 cm and diameter 20 cm) containing quartzite. The wheat plants were cultivated under controlled conditions (300 μmol photon m^{-2} s^{-1} of photosynthetic photon flux density (PPFD) light; 75–85 % of relative humidity; 25/20 °C of a continuous day/night temperature and a 12/12 h of day/night cycle, respectively). Hoagland nutrient solution was sprayed on quartzite every day (Hoagland and Arnon 1950). Fifteen days after the anthesis (grain filling stage), the plants were sprayed on leaves with an aqueous solution containing 100 mM GB, as described earlier in studies of uptake and translocation of GB in crop plants (Mäkelä et al. 1996). Control plants were sprayed with water. Three days later, the pretreated plants were subjected to heat and high light stresses (temperature 39 ± 2 °C; light 1,600 μmol photon m^{-2} s^{-1} of PPFD) for 1 and 2 h in the climate chamber. The flag leaves from all of the treated plants were sampled and analyzed immediately. The experiment was repeated at least three times under the same conditions.

Determination of chlorophyll content

Chlorophyll content was determined and calculated using the method proposed by Arnon (1949). The samples were homogenized with 10 mL of acetone (80 % v/v) using precooled pestle and mortar. Then the homogenate was centrifuged at $5{,}000 \times g$ for 10 min. The absorbance was

measured with a UV–Visible spectrophotometer at 663 and 645 nm (Beckman, CA, USA).

Chlorophyll *a* fluorescence parameters

Chlorophyll *a* fluorescence parameters were measured using a portable Chl fluorometer, (FMS-2, Hansatech, UK). F_v/F_m, represented the maximal photochemical efficiency. Leaves were kept in the dark for 20 min before measurement. The saturated flash intensity was 8,000 μmol m^{-2} s^{-1} to determine the maximum (F_m) fluorescence. F_v/F_m was calculated with the following formula: $F_v/F_m = (F_m - F_o)/F_m$, where F_o is initial fluorescence, F_m is maximum fluorescence, and F_v is variable fluorescence.

Photosynthetic gas-exchange parameters measurements

The measurements of photosynthetic gas exchange parameters including net photosynthetic rate (P_n) and stomatal conductance (g_s) were determined using a LI-6400 photosynthesis equipment according to the manufacture's recommendation (Li-cor, USA). The light source was used to set PPFD at 1,000 μmol m^{-2} s^{-1} for all measurements while reference CO_2 was set at 400 ppm. The measurements was carried out with fully expanded attached leaves, and lasted approximately 10 min until no significant recovery was observed.

Preparation of total RNA and real-time PCR quantification of *psbA* transcription

Total RNA was extracted from flag leaves by Trizol RNA extraction solution (Invitrogen, Inc., Carlsbad, CA, USA) following the manufacturer's recommendations. Approximately 3 μg of total RNA was reverse transcribed using an oligo (dT) primer and MMLV reverse transcriptase (Fermentas Life Sciences, MD, USA) according to the manufacturer's recommendations. After reverse transcription, the cDNA was quantified by real-time PCR assay. Primer set for the *psbA* gene (accession number NC002762) (psbA-F: 5′-GGAGGGGCAGCGATGAAGGC-3′ and psbA-R: 5′-GCCTGTGGGGTCGCTTCTGC-3′) was designed using the Primer Express 3.0 software (Applied BioSystems, Foster City, CA, USA). Each reaction was repeated at least three times. Data were normalized using the wheat 18S ribosomal RNA (18S rRNA) subunit genes as internal controls. The primers for 18S rRNA (18S-F: 5′-GTGACGGGTGACGGAGAATT-3′ and 18S-R: 5′-GACACTAATGCGCCCGGTAT-3′) were followed by Csiszár et al. (2012). Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Briefly, the additive effect of concentration, gene and replicate was minimized by subtracting the C_t number of the target gene from that of the reference gene, which yielded ΔC_t. This value was subtracted from all other ΔC_t values, which yield the $\Delta\Delta C_t$.

Determination of GB content

The GB content was determined using a high-performance liquid chromatography (HPLC) (LC-6A; Shimadzu, Kyoto, Japan) according to the procedure described by Ma et al. (2006). GB concentration was quantified by comparing the peak surface areas with those obtained with pure glycinebetaine standards.

Determination of ABA content

Before quantitative determination, the ABA was extracted from wheat leaves according to Vaseva et al. (2010). Briefly, 1 g of fresh leaf material was ground in extraction solution containing 80 % methanol (v/v), then homogenized overnight at 4 °C with constant shaking. After centrifugation at 15,000 *g* for 20 min, the supernatant was subjected to ABA determination by enzyme-linked immuno sorbent (ELISA) assay. The ABA concentration was measured at 450 nm with a UV–Vis spectrophotometer (Thermo Fisher Inc. Waltham, MA, USA) using an abscisic acid immunoassay kit (Yanjin Biological, Shanghai, China) according to the manufacturer's protocol.

Statistical analysis

Each pot was treated as one replicate, all treatments were repeated at least three times with three plants. All the figures were drawn using Excel software (Microsoft Corporation, USA). Statistical analysis was conducted using the SPSS Statistics 18.0 software (IBM Corporation, NY, USA). One-way analysis of variance (ANOVA) was done with all the data to confirm the variability of data and validity of the results. Differences between the means among treatments were compared using Duncan's multiple range tests at 0.05 probability levels.

Results

Chlorophyll content

As shown in Fig. 1, the chlorophyll content in wheat leaves decreased significantly under heat and high light stresses compared to the control. Compared to the non-stressed control, chlorophyll content was decreased by 24 and 37 % at 1 and 2 h, respectively. Exogenous GB application maintained significantly higher levels of chlorophyll

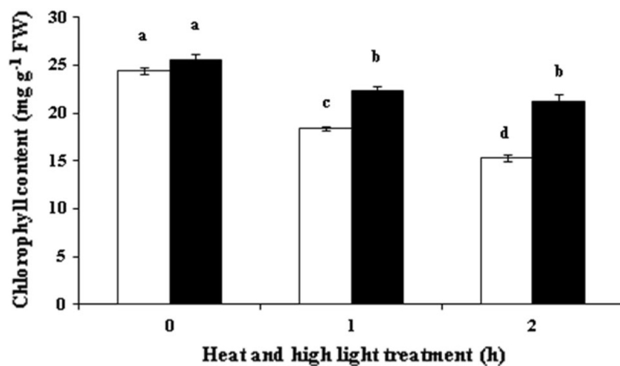

Fig. 1 Effect of GB on the chlorophyll content in wheat flag leaves under heat and high light stresses. The wheat plants were grown under 25 ± 2 °C and 300 μmol m^{-2} s^{-1} of PFD before stress (non-stressed). The stressed plants were exposed to 39 ± 2 °C and 1,600 μmol m^{-2} s^{-1} of PFD (heat and high light stressed). The *open rectangle* represented the wheat plants treated by double distilled H_2O (ddH_2O); the *solid rectangle* represented the wheat plants pretreated by 100 mM GB. The values are mean ± SE ($n = 3$). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test

content (21 and 38 % at 1 and 2 h of heat and high light stresses, respectively) on the chlorophyll content, as compared to the plants subjected to heat and high light stresses without GB.

Chlorophyll fluorescence and PSII activity

Heat and high light stresses led to significant decreases in F_v/F_m, which were 29 and 46 % lower than non-stressed controls (Fig. 2a). Foliar application of GB was able to retard the decreases in F_v/F_m. 100 mM exogenous GB could maintain the F_v/F_m at the same level as non-stressed control ($P > 0.05$) after 1 h of stress and kept 82 % after 2 h, which were 37 and 56 % higher than respective stressed alone wheat plants (without GB). Similarly, foliar application of GB could also retard the decline of P_n in wheat plants subjected to heat and high light stresses, which were 49 and 66 % higher than stressed alone wheat plants (without GB) (Fig. 2b).

Relative expression level of *psbA* gene

Heat and high light stresses resulted in a significant decrease in relatively transcriptional expression level of the *psbA* gene, which were 81 and 84 % lower than non-stressed control after 1 and 2 h of stress exposure, respectively (Fig. 3). Foliar GB supplementation significantly retarded the decrease of *psbA* transcription, and was 2.44 fold and 2.15 fold higher than the stressed alone wheat plants after 1 and 2 h of heat and high light stresses, respectively.

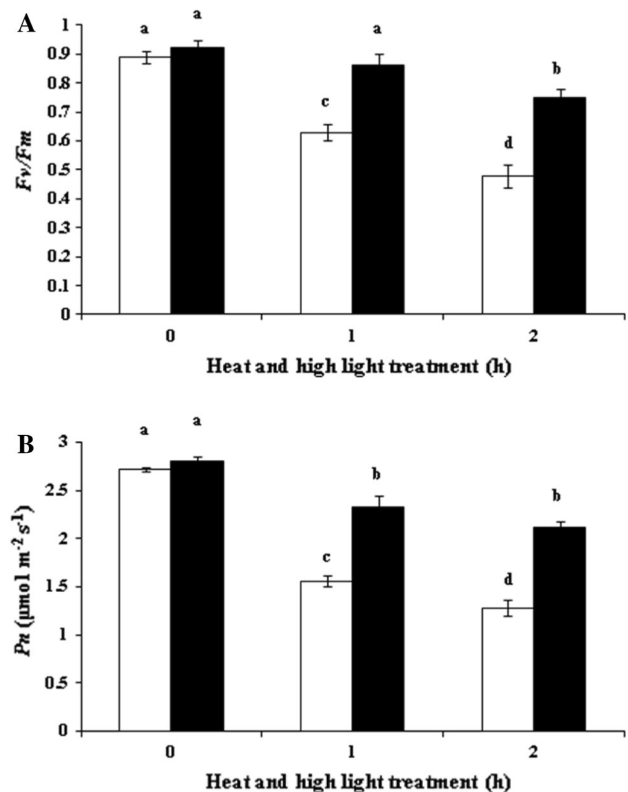

Fig. 2 Effects of GB on the F_v/F_m (**a**), P_n (**b**) in wheat flag leaves under heat and high light stresses. The wheat plants were grown under 25 ± 2 °C and 300 μmol m^{-2} s^{-1} of PFD before stress (non-stressed). The stressed plants were exposed to 39 ± 2 °C and 1,600 μmol m^{-2} s^{-1} of PFD (heat and high light stressed). The *open rectangle* represented the wheat plants treated by ddH_2O; the *solid rectangle* represented the wheat plants pretreated by 100 mM GB. The values are mean ± SE ($n = 3$). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test

Stomatal conductance

Guard cells modulate leaf transpiration and CO_2 uptake by changing stomatal conductance (Kopka et al. 1997). As shown in Fig. 4, heat and high light stressed wheat plants showed a significant reduction in the stomatal conductance by 37 and 55 % after 1 and 2 h of stress exposure, respectively, compared with non-stressed control treatments. Whereas foliar GB supplementation could not only improve the stomatal conductance without stress exposure, but also prevent again the stress-induced decline in stomatal conductance, which was 33 and 51 % higher than stressed alone wheat plants, upon 1 and 2 h of heat and high light treatment, respectively.

GB and ABA content

Exogenous GB application was able to significantly increase the GB content of wheat leaves, which was 76 %

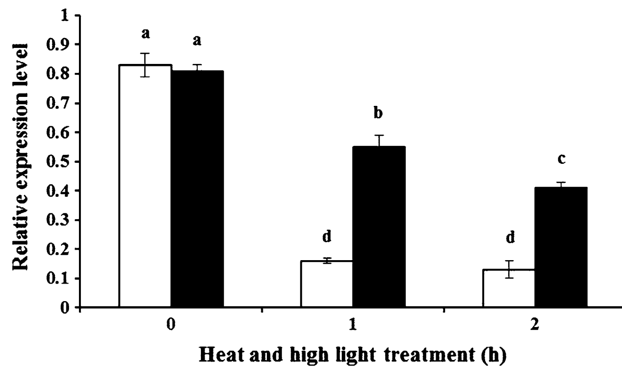

Fig. 3 Effect of GB on relative expression level of *psbA* gene in wheat flag leaves under heat and high light stresses. The wheat plants were grown under 25 ± 2 °C and 300 μmol m^{-2} s^{-1} of PFD before stress (non-stressed). The stressed plants were exposed to 39 ± 2 °C and 1,600 μmol m^{-2} s^{-1} of PFD (heat and high light stressed). The *open rectangle* represented the wheat plants treated by ddH_2O; the *solid rectangle* represented the wheat plants pretreated by 100 mM GB. The values are mean ± SE ($n = 3$). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test

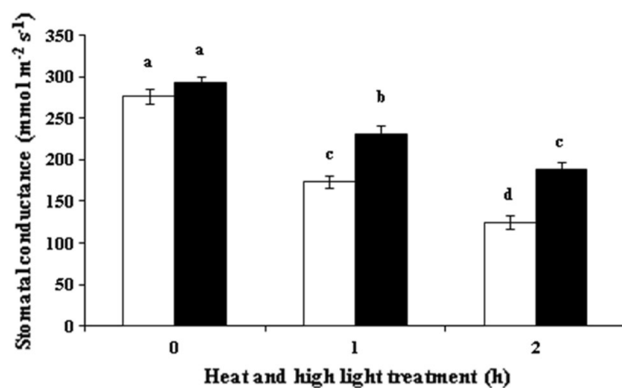

Fig. 4 Effects of GB on the stomatal conductance (g_s) in wheat flag leaves under heat and high light stresses. The wheat plants were grown under 25 ± 2 °C and 300 μmol m^{-2} s^{-1} of PFD before stress (non-stressed). The stressed plants were exposed to 39 ± 2 °C and 1,600 μmol m^{-2} s^{-1} of PFD (heat and high light stressed). The *open rectangle* represented the wheat plants treated by ddH_2O; the *solid rectangle* represented the wheat plants pretreated by 100 mM GB. The values are mean ± SE ($n = 3$). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test

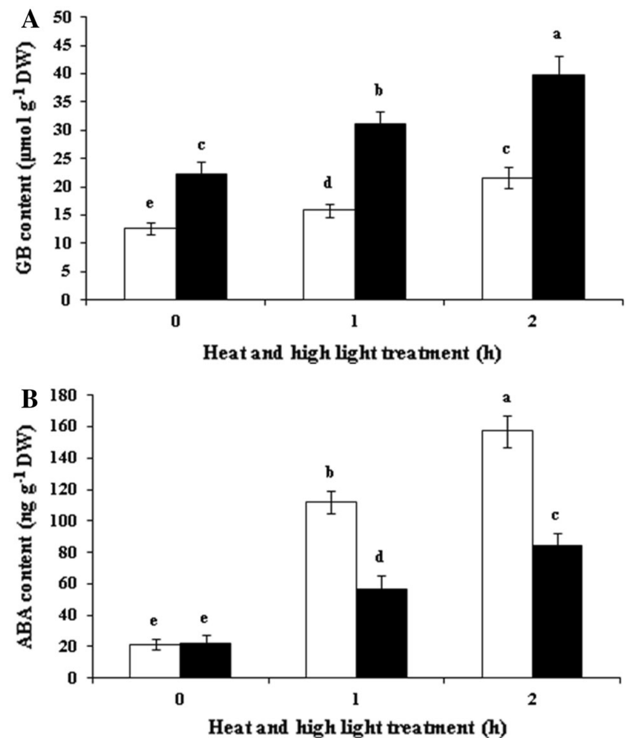

Fig. 5 Effects of GB on the endogenous accumulation of GB (**a**) and ABA (**b**) in wheat flag leaves under heat and high light stresses. The wheat plants were grown under 25 ± 2 °C and 300 μmol m^{-2} s^{-1} of PFD before stress (non-stressed). The stressed plants were exposed to 39 ± 2 °C and 1,600 μmol m^{-2} s^{-1} of PFD (heat and high light stressed). The *open rectangle* represented the wheat plants treated by ddH_2O; the *solid rectangle* represented the wheat plants pretreated by 100 mM GB. The values are mean ± SE ($n = 3$). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test

higher than wheat plants without GB supplementation before stress treatment (Fig. 5a). With the increase of heat and high light stresses, the GB concentration increased whether it had been pretreated with GB or not. Moreover, GB-treated plants always kept a higher GB level than non-GB treated wheat plants. The ABA content also increased along with increasing heat and high light stresses in either GB pretreated or not wheat plants (Fig. 5b). However, foliar GB supplementation could significantly retard the ABA increase, which were 50 and 46 % lower than stressed alone wheat plants, after 1 and 2 h of heat stress combined with high irradiance, respectively.

Discussion

When higher plants were suffering from abiotic stress, there normally happened a reduction in chlorophyll biosynthesis or improvement in degradation (Dall'Osto et al. 2006), and reduction in photosynthetic activity, which can be due to or lead to photoinhibition (Allakhverdiev et al. 2008; Tyystjärvi 2013). Our results indicated that heat and high light stresses significantly decreased the chlorophyll content in wheat leaves (Fig. 1). During the grain filling stage, the improved decrease of chlorophyll content under high light and heat stresses was more probably caused by the enhancement of chlorophyll degradation than decrease of biosynthesis. Thus, it could be indicated that the GB-retarded decrease of chlorophyll content might had a great relationship with its protection effect on the degradation of chlorophyll under heat and high light stresses.

PSII is believed to play a key role in the response of leaf photosynthesis to environmental perturbations (Murata et al. 2007). The moderate irradiance plays an important role in the heat tolerance of PSII function in wheat by offering light-driven electron flow (Marutani et al. 2012). However, higher irradiance single or combined with other abiotic stress could also cause additional photoinhibition in many higher plants (Chen et al. 2012; Ma et al. 2006; Vass 2012). The strong protective effect of GB on structure and function of the complex of PSII photosystem against different environmental stresses has been well established in vitro (Allakhverdiev et al. 2008). F_v/F_m and P_n are usually used as sensitive indicators of plant photosynthetic performance and represent the measure of the functional status of the oxygen evolving complex (Baker 2008). In the present study, when grain filling wheat plants were exposed to heat stress combined with high irradiance, there were shown significant declines in F_v/F_m and P_n, but the declines were inhibited by foliar spraying of GB (Fig. 2).

The extent of photoinhibition in plants suffering from abiotic stress depends on the balance between the rate of photodamage and the rate of repair (Murata et al. 2007). Previous studies indicated that, GB accumulation, whether due to exogenous application or due to genetic engineering, does not protect PSII from photodamage. Thus, the protective effect of GB on PSII activity was considered to be due to an enhancement of PSII repair after the heat-enhanced photoinhibition (Yang and Lu 2006; Yang et al. 2007). Previous studies upon wheat plants have shown that GB over-accumulation enhances the tolerance of the photosynthetic apparatus to drought and heat stresses (Wang et al. 2010a). Our results also confirm that GB accumulation by exogenous application increased the tolerance of PSII to heat and high light stresses (Fig. 2). Thus, we have further investigated the possible mechanisms responsible for the alleviation of photoinhibition in the GB supplemented wheat plants under heat and high light stresses.

It is known that D1 protein is one of the most important componential and functional proteins in the PSII complex, and is one of the most vulnerable component to environmental stress (Edelman and Mattoo 2008; Yamamoto 2001), in that abiotic stress inhibits PSII repair during photoinhibition by interfering with the de novo synthesis of D1 protein (Chen and Murata 2011). GB mediated alleviation of photoinhibition under heat and high light stresses has shown close correlationship with the enhancement of stability of D1/D2/Cytb559 complex in spinach (Allakhverdiev et al. 2003). In the transgenic tomato, GB accelerated the accumulation of D1 protein (Li et al. 2014). The *psbA* gene is responsible for the reproduction of new D1 proteins for the replacement of the portion injured by stress, and plays an important role in D1 protein turnover during stress resistance (Mulo et al. 2009; Wang et al. 2011). In present research, the transcription of *psbA* gene was suppressed by heat and high light stresses, but was alleviated by the in vivo GB accumulation (Fig. 3). The induced *psbA* transcription may enhance the de novo synthesis of the new copies of D1 protein, accelerate the D1 protein accumulation, and then improve the turnover of it and dissipate more excess light energy (Mulo et al. 2012), which is all beneficial for the improvement of PSII tolerance under heat and high light stresses.

The opening or closure of the stomata is traditionally considered as being tightly regulated as reflection to abiotic stress (Roelfsema and Hedrich 2005). When temperature and irradiance is suitable, wheat plants open their stomata to transfer water and minerals from the roots to the shoots, and to exchange gases. The stomatal movement is affected by several environmental stimuli, such as water deficit, CO_2 concentration, high irradiance and plant hormones, including ABA, salicylic acid (SA) and ethylene (Wilkinson and Davies 2010). The role of ABA is accepted to play an important role in stomatal closure during drought stress (Wilkinson and Davies 2010; Miura et al. 2013). The present data also showed that the ABA was accumulated in wheat leaves when heat and high light stresses were imposed (Fig. 5). Similar results have been reported in *Arabidopsis* that, both inhibitor data and genetic data suggested ABA were actually used in vivo to protect again heat-induced oxidative stress (Larkindale and Knight 2002). However, although the PSII activity in wheat leaves was protected by foliar application of GB, present data indicated that exogenous GB inhibited the accumulation of endogenous ABA under heat and high light stresses (Fig. 5). Correspondingly, and stomatal closure were inhibited as a result (Fig. 4). Similar inhibition effect of exogenous GB application on ABA accumulation was also shown in papaya seedlings during water-deficit stress (Mahouachi et al. 2012).

In summary, the present study showed that foliar application of GB was able to decrease the susceptibility of the PSII in wheat leaves to photoinhibition that is caused by heat stress combined with high irradiance; this is mainly through accelerating the turnover of D1 protein in PSII by the inducement of the *psbA* gene transcription, as well as the improvement of stomatal conductance. Further work is required to elucidate the mechanism of GB-induced stomatal movement and PSII photoprotection.

Author contribution Yuexia Wang and Huijie Zhao worked on the experimental design and fabrication of this manuscript. Yuexia Wang, Shuchun Liu, Huanling Zhang, Huashan Liu contributed on the performance of experiments. Yuexia Wang and Yidan Zhao contributed to the data analysis.

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