



## Research article

# Redox and thylakoid membrane proteomic analysis reveals the *Momordica charantia* L.) rootstock-induced photoprotection of cucumber leaves under short-term heat stress

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## ABSTRACT

Heat stress adversely affects plant physiological and metabolic processes and is considered an important constraint on crop growth and productivity in agriculture worldwide. Grafting techniques are capable of mitigating various stresses. Here, compared with self-grafted cucumbers subjected to 42 °C heat stress for 24 h, we found that *Momordica*-grafted cucumbers exhibited higher cytomembrane thermostability, less photoinhibition reflected by their chlorophyll fluorescence, and a reduction in oxidative stress. To better understand the mechanism, optimized Blue-Native/SDS-PAGE two-dimensional electrophoresis (2-DE) was firstly applied to entire thylakoid membrane of grafted cucumbers, and 25 significantly differential accumulated protein spots were identified by MALDI-TOF/TOF MS analysis. The proteomic analysis revealed that high temperatures suppressed the accumulation of 13 proteins in self-grafted cucumbers, while *Momordica* rootstock stimulated the accumulation of 12 of these proteins. The transcriptional analysis indicated that grafting onto *Momordica* significantly increased the expression of genes that encode the photosystem II subunit S (PsbS) and minor light-harvesting complexes (CP24, CP26 and CP29.1), which are closely associated with non-photochemical quenching (NPQ) after heat shock. Immunoblotting for PsbS corroborated the *Momordica*-induced acceleration of heat dissipation. Taken together, *Momordica* rootstock alleviated heat-induced photoinhibition by maintaining intracellular redox homeostasis, stabilizing the protein library of the thylakoid membrane and modulating NPQ in the scions.

## 1. Introduction

As the Earth warms, land surface temperatures are estimated to rise by 1.4–3.0 °C by 2050 and 2–4.5 °C by 2100 along with more frequent extremely high temperatures (Brestic et al., 2016; Pounds et al., 2006). Heat stress seriously jeopardizes the performance of plants, and thereby causes devastating crop yield losses. It is well established that photosynthesis is the most thermally labile process in higher plants (Haque

et al., 2014; Hou et al., 2016). Photoinhibition occurs ubiquitously when various abiotic stresses unbalance the harvesting and utilization of light even at relatively low light intensities (Cazzaniga et al., 2013; Gururani et al., 2015), and concomitantly, the over-production of reactive oxygen species (ROS) destroys the photosynthetic apparatus, forming a vicious circle (Yoshida and Hisabori, 2016). Moreover, the light reactions of photosynthesis are sensitive to multiple abiotic stresses which could induce considerable remodeling of the thylakoid

**Abbreviations:** 2-DE, two-dimensional gel electrophoresis; APX, ascorbate peroxidase; ASA, ascorbic acid; DHA, dehydroascorbic acid; DHAR, dehydroascorbate reductase; Fm, maximal fluorescence; Fm', light adapted maximal fluorescence; F<sub>0</sub>, initial fluorescence; F<sub>s</sub>, steady-state fluorescence; F<sub>v</sub>/F<sub>m</sub>, maximum photochemistry efficiency of PSII; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; LHCs, light-harvesting complexes; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; NPQ, non-photochemical quenching; O<sub>2</sub><sup>-</sup>, superoxide anion; PS, photosystem; PsbS, photosystem II subunit S; qE, energy-dependent nonphotochemical quenching; qN, non-photochemical quenching coefficient; RC, reaction center; REL, relative electrolyte leakage; ROS, reactive oxygen species; ΦPSII, actual photochemical efficiency of PSII

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membrane (Shan et al., 2015). It has been proposed that photosystem II (PSII) has a higher susceptibility to superoxide-derived ROS than PSI (Krieger-Liszka et al., 2011). Similarly, PSII is widely considered the critical site of thermal-induced photoinhibition, which concurs with the proposition that the acceptor-side of PSII could be principally suppressed by short-term heat stress (Allakhverdiev et al., 2008; Yan et al., 2011).

In recent years, massive efforts have been made in elevating the thermotolerance of plants. For instance, high-tech genetic breeding achieved by hybridization and recombinant/transgenic formation has cultivated numerous heat-resistant plant varieties (Geng et al., 2016; Mangrauthia et al., 2017). An exogenous application of phytohormones and plant growth regulators, such as ABA, melatonin and spermidine, also enhances plant tolerance to heat shock (Aydogan et al., 2017; Sang et al., 2017; Xu et al., 2016). More importantly, the plant-grafting technique which originated from Japan with the primitive motivation to reduce the incidence of soil-borne pathogens, was demonstrated to successfully mitigate external environmental stresses, especially abiotic stresses (Gregory et al., 2013; Ntatsi et al., 2014). Nevertheless, little empirical and theoretical information regard in the mechanism of heat-tolerance improvement triggered by grafting onto other-species rootstock has been accumulated. Remarkably, hydrogen peroxide ( $H_2O_2$ ) is elucidated to regulate thermal resistance and root-originated ABA transmits to the shoot via the xylem to enhance the abundance of heat shock proteins and/or stress-related genes after heat exposure when luffa is selected as a rootstock (Li et al., 2014a, 2014b). Moreover, *csa-miR159b* is identified by microarray and genetic analyses to mediate luffa rootstock-induced modification of heat tolerance (Li et al., 2016b). Additionally, grafting onto tolerant genotypes usually attenuates the thermal stress indicated by photoinhibition and ROS over-accumulation because those plants maintain their redox status better than self-grafted or non-grafted plants, which causes less damage to their photosynthetic apparatus in turn (Rivero et al., 2010).

Cucumber is a cost-effective vegetable species in protected cultivation, and it is vulnerable to heat stress. A transient elevation in temperature is capable of disrupting intracellular redox homeostasis and extremely suppressing photosynthesis in cucumbers (Ding et al., 2016). The performance of the shoot is closely correlated with its rootstock genotype (Lee et al., 2010). However, current studies involving the rootstock of cucumbers are far from sufficient because they concentrate on salt and cold tolerance and usually use pumpkin species as rootstocks (Xing et al., 2015). Xu et al. (2018) took some breakthroughs by grafting cucumber onto heat resistant *Momordica* (Mc), and they hypothesized that the alleviation in growth inhibition caused by heat stress might be relevant to rootstock-induced photosynthesis improvement.

Photoprotection involves diverse strategies, such as the non-photochemical quenching (NPQ) mechanism whose dominant component is energy-dependent non-photochemical quenching (qE), the conformation interconversion and reorganization of LHCs, and cyclic electron transport around the PSI (Johnson et al., 2011; Ruban et al., 2012). Among them, the photosystem II subunit S (PsbS) plays a crucial role in sensing the proton gradient across the thylakoid membrane by which qE regulates the detachment or the aggregation of PSII antenna to balance the capture and utilization of light under stress (Krishnan et al., 2017). In order to better understand the Mc-induced photoprotective mechanism under heat shock, as well as to examine whether PsbS-related NPQ is involved in this process, we investigated the alleviating effects of Mc on heat stress from the perspective of redox status and the levels of thylakoid membrane proteins.

## 2. Materials and methods

### 2.1. Plant materials and treatments

Cucumber (*Cucumis sativus* L. cv. Jinchun No. 2, obtained from

Tianjin Kernel Cucumber Research Institute, China) was used as the scions, and *Momordica* (*Momordica charantia* L. cv. Changlv, obtained from Guangdong Academy of Agricultural Sciences, China) was used as the rootstocks. Cleft grafting was used in this study, and self-grafted plants were included as controls. Seeds of the rootstock were grown in plastic pots filled with cultivating medium 7 days earlier than the seeds of the scion. These seedlings were cultivated in a growth chamber (RDN-560E-4; Dongnan Instrument, Ningbo, China), in which the conditions were controlled as follows: air temperature, 28/18 °C (day/night); maximum photosynthetic photon flux density (PPFD) (LED light source; Dongnan Instrument, Ningbo, China),  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ ; relative humidity, 70–75%; and light/dark photoperiod, 14/10 h (day/night).

Cleft grafting was performed when the cotyledons of the scions and the first true leaves of the rootstocks had fully expanded. Grafted plants were transferred to a small plastic arched shed. They were maintained at a temperature above 25 °C and a relative humidity between 85% and 100% for 7 days until the graft union had completely healed. Next, grafted plants were transferred to a growth chamber. The seedlings were not treated until the fourth true leaves completely expanded: (1) self-grafted plants treated at 28 °C, Cs-28; (2) *Momordica*-grafted plants treated at 28 °C, Mc-28; (3) self-grafted plants treated at 42 °C for 24 h, Cs-42; and (4) *Momordica*-grafted plants treated at 42 °C for 24 h, Mc-42. All of the following experimental procedures were performed using the third fully expanded leaves, numbered basipetally. Leaf samples were harvested at 24 h, frozen in liquid nitrogen and stored at  $-80 \text{ }^\circ\text{C}$  until further analysis.

### 2.2. Chlorophyll fluorescence and pigment determination

The chlorophyll fluorescence was detected with IMAGING-PAM (Walz, Effeltrich, Germany) according to Lu et al. (2003) with modifications. Imaging Win software (Walz, Effeltrich, Germany) was used to obtain data and fluorescence images of leaves. Plants were dark-adapted for 30 min before the initial fluorescence  $F_0$  was obtained, and then, the maximal fluorescence ( $F_m$ ) was determined by a saturating pulse ( $12000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 0.8 s). The actinic illumination of  $450 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  followed by a second saturating pulse was used to measure the steady-state fluorescence ( $F_s$ ) and the light adapted maximal fluorescence ( $F_m'$ ). The maximum photochemical efficiency of PSII ( $F_v/F_m$ ), ( $F_0$ ), actual photochemical efficiency of PSII ( $\Phi\text{PSII}$ ), and non-photochemical quenching coefficient (qN) were calculated by data from the software.

The chlorophyll (Chl) content and the carotenoid content were measured as previously described (Arnon, 1949). Briefly, leaf samples (0.1 g) were shredded and soaked in 20 mL of ethanol/acetone/distilled water (4.5/4.5/1, v/v/v) in darkness until completely whitened, and then, the absorbance was measured at 645 nm and 663 nm using a UV spectrophotometer (T6; Beijing Purkinje General Instrument, Beijing, China).

### 2.3. Membrane thermostability and ROS analysis

The malondialdehyde (MDA) content and relative electrolyte leakage (REL) were measured by methods described previously with slight modification (Hodges et al., 1999; Lutts et al., 1996). To determine the content of MDA, 0.5 g leaves was homogenized in 5 mL 5% (w/v) trichloroacetic acid. The homogenate was centrifuged at 15000 g for 10 min. Then 2 mL 0.67% TBA was added into each centrifuge tube containing 2 mL supernatant. The mixture was heated at 100 °C for 30 min before re-centrifuging at 12000 g for 15 min followed by the absorbance measurement.

$H_2O_2$  level was estimated according to the protocol reported by Su et al. (2005) with modifications. Leaf samples were homogenized with 2 mL pre-cooling acetone, and the mixture was centrifuged at 10000 g for 15 min to obtain  $H_2O_2$  extract. Then 50 mL 20% titanium

tetrachloride and 0.1 mL strong ammonia were added to each of 0.5 mL extract, after which the solution was thoroughly mixed and centrifuged to get peroxide-Ti complexes. After a four-time wash using acetone, the final precipitate was dissolved by 3 mL 2 M H<sub>2</sub>SO<sub>4</sub>. Finally, the H<sub>2</sub>O<sub>2</sub> content was quantified by spectrophotometric analysis at 415 nm. The generation rate of superoxide anion (O<sub>2</sub><sup>•-</sup>) was measured via analyzing the absorbance at 530 nm, and then, it was calculated by a [NO<sub>2</sub><sup>-</sup>]-based standard curve (Eltner and Heupel, 1976).

#### 2.4. Ascorbate-glutathione (ASA-GSH) cycle analysis

The content of ascorbate and glutathione was measured as previously described (Costa et al., 2002; Griffith, 1980). For detecting the activity of ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2), dehydroascorbate reductase (DHAR, EC 1.8.5.1) and monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), leaf samples (0.3 g) were ground with 2 mL of ice-cold buffer containing 50 mM PBS (pH 7.8), 0.2 mM EDTA, 2 mM AsA, and 2% (w/v) polyvinylpyrrolidone. The homogenates were centrifuged at 4 °C for 20 min at 12000 g, and the resulting supernatants were used for the determination of enzymatic activity as previously described (Nakano and Asada, 1981). The protein concentration was determined with bovine serum albumin as the standard.

#### 2.5. Protein extraction and separation

The third leaves from plants in the 4-leaf stage were harvested after treatment, and intact chloroplasts were immediately isolated for a more integrated membrane system depending on extraction and suspension buffers containing 330 mM sorbitol, 50 mM 2-(N-Morpholino) ethanesulfonic acid and 2 mM ASA. Thylakoid fractions were prepared from the isolated chloroplasts by osmotic rupture. After centrifugation at 4 °C at 14000 g for 3 min, the pellets containing the thylakoid membranes were resuspended, and the Chl concentration of the membranes was detected spectrophotometrically as previously described. The thylakoid membranes (15 µg Chl at 1 mg Chl/mL) were solubilized with 2% (w/v) n-Dodecyl-beta-D-maltoside and incubated at 4 °C for 30 min with gentle agitation. Then, the membrane proteins were extracted by centrifugation (15000 g) at 4 °C for 10 min and were stored at -20 °C.

Blue-Native/SDS-PAGE was carried out with a 4% spacer gel and a 5–12% separation gel filled by a gradient mixing device, after which 20 µL of the protein sample and pre-equipped marker were separately added. A constant voltage of 100 V and 180 V were applied to the stacking gel and the separating gel using PROTEAN II xi Cell System (Bio-Rad, Hercules, CA), respectively. As previously described, the gel lanes were immediately equilibrated in 5 mL of equilibration buffer for 30 min, and then, they were sliced for 2D-SDS-PAGE (Jarvi et al., 2011; Wittig et al., 2006).

#### 2.6. Spots analysis

Gels were stained with Coomassie Brilliant Blue (R-250), decolorized by a destaining solution, and scanned with Image Scanner III (GE Healthcare, San Francisco, CA). Protein spots were quantified using Image Master™ 2D Platinum software (version 6.0; GE Healthcare, San Francisco, CA). Spots with at least a 1.5-fold quantitative and reproducible change in volume percentage (vol. %, the ratio of the spot volume to that of the entire gel) were regarded as being significantly regulated.

#### 2.7. Protein identification

Enzymatically digested in-gel proteins which were detected differentially accumulated were analyzed using an ABI 5800 proteomic analyzer MALDI-TOF/TOF system (Applied Biosystems, Foster City, USA). The laser source was a Nd:YAG laser with a wavelength of

349 nm. The acceleration voltage was 2 kV. Data was collected in a mode of positive ion and automatically acquired with 1000 laser shots per spectrum. The detection range of monoisotopic peak mass was 800–4000 Da, and then primary ions with signal-to-noise ratio higher than 50 were chosen for secondary mass spectrometry (MS/MS) analysis. Averaged MS/MS spectra were obtained in a positive ion mode with a 2 kV collision energy (2500 times). The MS and MS/MS spectral data were matched by searching the NCBI\_Momordica charantia database (29025 protein sequences; download in 2017/12/22), the NCBI\_Cucumis sativus database (51150 protein sequences; download in 2017/3/15) and the NCBI\_Cucumis database (83365 protein sequences; download in 2017/2/24) using the software MASCOT version 2.2 (Matrix Science, London, UK). Database searching parameters were set as follows: one missed cleavage of trypsin was allowed; carbamoylmethyl and oxidation of methionine were set as fixed modification and variable modification, respectively; peptide and fragment mass tolerance were set to ± 100 ppm and ± 0.4 Da, respectively; the minimum protein score confidence interval for MS/MS data was set to 95%.

#### 2.8. Total RNA extraction and qRT-PCR analysis

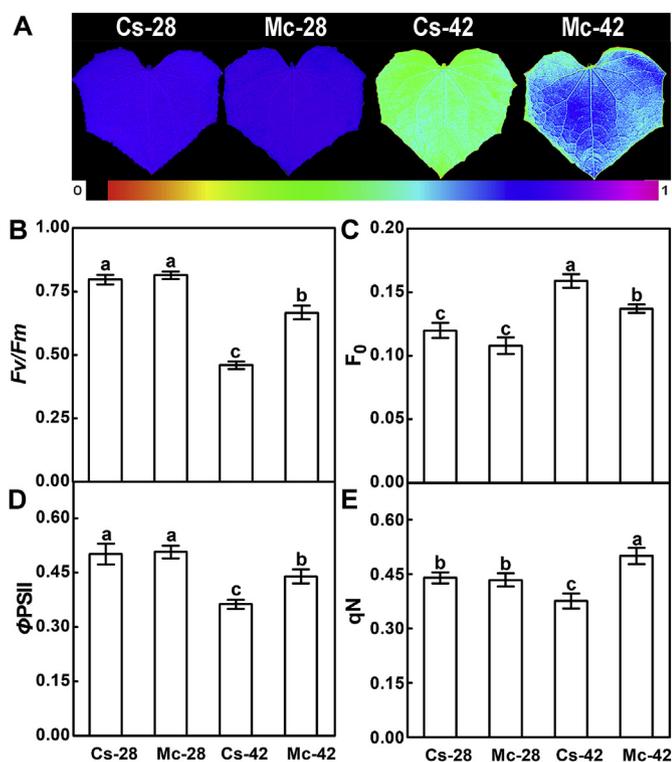
Total mRNA was isolated from cucumber leaves as described in the TRI reagent protocol (Axygen, Union City, CA). One microgram of total RNA was used to reverse transcribe a cDNA template using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). qRT-PCR assays were conducted in a StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (BioRad, Hercules, CA). The PCR conditions consisted of denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s and extension at 72 °C for 30 s. The cucumber *actin* gene was used as an internal control. Gene-specific primers were designed according to the cDNA sequences and were listed in Table S1. Relative expression was calculated using the 2<sup>-ΔΔCt</sup> method as previously described (Livak and Schmittgen, 2001).

#### 2.9. Immunoblot analysis

For immunoblotting, protein was extracted from 0.5 g of cucumber leaves using a buffer containing 30 mM Tris-HCl (pH 8.7), 1 mM EDTA-Na<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 0.7 mM sucrose, 1 mM Phenylmethanesulfonyl fluoride, 1 mM ASA and 1 mM DTT. After protein separation using 12% SDS-PAGE, proteins on the SDS-PAGE gel were transferred onto a 0.22 µm polyvinylidene fluoride (PVDF) membrane by Trans-Blot SemiDry (BioRad, Hercules, CA). Next, the membrane was blocked with 5% non-fat dry milk for 1 h and incubated with a rabbit anti-PsBS polyclonal antibody (Agriserä, Vännäs, Sweden). After incubation with a goat anti-rabbit HRP-linked antibody (Origene, Rockville, MD), the membrane was analyzed using a SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, 34080) following the manufacturer's instructions.

#### 2.10. Statistical analysis

At least 3 independent biological replicates were used for each analysis. Analysis of variance (ANOVA) was used to test for significance, and significant differences ( $P < 0.05$ ) between treatments were determined using Duncan's multiple range tests in SPSS 22.0 software (SPSS, Chicago, IL). Data in clustering heat maps originated from the standardized relative abundance of genes and the vol. % of protein spots after log<sub>2</sub> and log<sub>10</sub> transformation (Table S2), respectively, before using Heatmap Illustrator software (version 1.0; Wuhan, China).



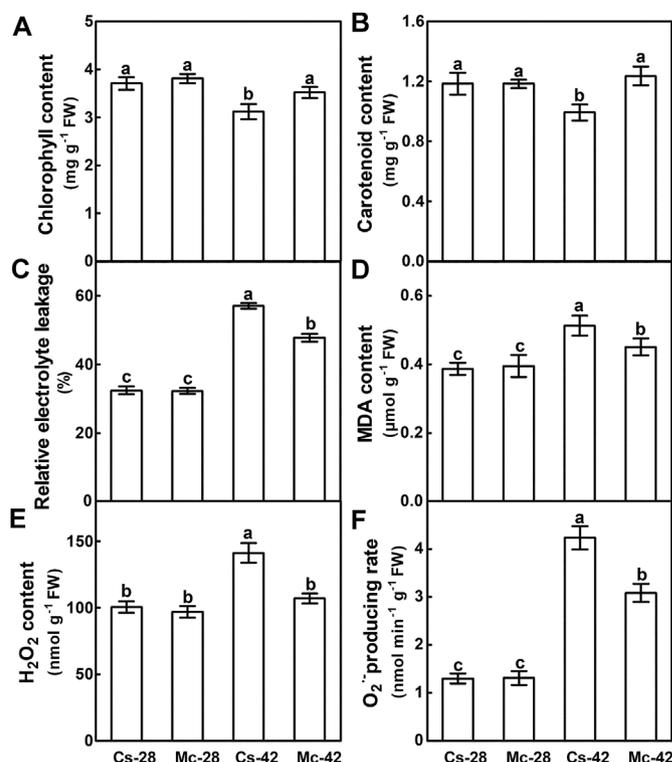
**Fig. 1.** Effects of *Momordica* rootstock on the chlorophyll fluorescence of cucumbers after a 24 h-heat treatment. Images of the maximum photochemical efficiency of PSII ( $F_v/F_m$ ) (A and B), initial fluorescence ( $F_0$ ) (C), actual photochemical efficiency of PSII ( $\Phi_{PSII}$ ) (D), and the non-photochemical quenching coefficient ( $q_N$ ) (E) with actinic illumination of  $450 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  using a chlorophyll fluorescence imaging technique. The color scale indicates values from 0 (black) to 1 (pink). Values are shown as the mean  $\pm$  SE. ( $n = 5$ ). Different letters represent statistically significant differences at  $P < 0.05$  according to Duncan's multiple range tests. Cs-28, self-grafted cucumber seedlings treated at  $28^\circ\text{C}$ ; Mc-28, seedlings grafted onto *Momordica* treated at  $28^\circ\text{C}$ ; Cs-42, self-grafted cucumber seedlings treated at  $42^\circ\text{C}$ ; Mc-42, seedlings grafted onto *Momordica* treated at  $42^\circ\text{C}$ . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

### 3. Results

#### 3.1. Effects of *Mc*-rootstock on the photosystem susceptibility to heat

To investigate whether grafting onto the rootstock of the thermo-tolerant species *Mc* could enhance heat stress tolerance in cucumber (*Cs*) plants, we first compared the photosystem susceptibility in cucumber leaves grafted onto its own root to those grafted onto *Mc* rootstock under heat stress. As shown in Fig. 1A and B, no significant difference was observed in  $F_v/F_m$  between plants grafted onto *Cs* and *Mc* under normal conditions. Although  $F_v/F_m$  in both self-grafted and *Mc*-grafted plants significantly decreased under heat stress,  $F_v/F_m$  in *Mc*-grafted plants was 45.0% higher than that in self-grafted plants at  $42^\circ\text{C}$  (Fig. 1B). Heat stress increased the value of  $F_0$  in self- and *Mc*-grafted plants (Fig. 1C). In contrast, the value of  $F_0$  in *Mc*-grafted plants was significantly lower than that in self-grafted plants. After heat stress for 24 h, the value of  $\Phi_{PSII}$  in the plants grafted onto its own roots and *Mc* roots decreased by 27.6% and 13.3%, respectively, in comparison to their own control plants (Fig. 1D). More importantly, although no apparent difference in the value of  $q_N$  was observed between *Mc*-28 and *Cs*-28 plants, there was a conspicuous increase in the value of  $q_N$  in *Mc*-grafted plants under heat stress (Fig. 1E).

The chlorophyll content was not significantly different between self-grafted and *Mc*-grafted plants at  $28^\circ\text{C}$ , but the chlorophyll content in



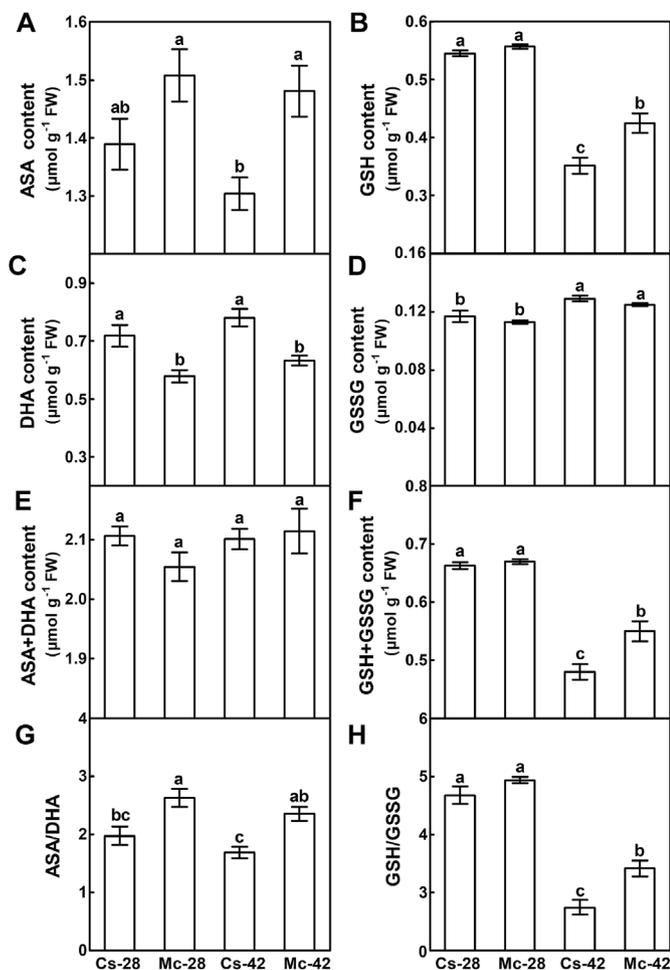
**Fig. 2.** Effects of *Momordica* rootstock on the pigment composition (A and B), membrane thermostability (C and D) and the production of ROS (E and F) of cucumbers after heat treatment for 24 h. Values are shown as the mean  $\pm$  SE. ( $n = 6$ ). Different letters represent statistically significant differences at  $P < 0.05$  according to Duncan's multiple range tests. MDA, malondialdehyde; Chl, chlorophyll; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; O<sub>2</sub><sup>·-</sup>, superoxide anion; Cs-28, self-grafted cucumber seedlings treated at  $28^\circ\text{C}$ ; Mc-28, seedlings grafted onto *Momordica* treated at  $28^\circ\text{C}$ ; Cs-42, self-grafted cucumber seedlings treated at  $42^\circ\text{C}$ ; Mc-42, seedlings grafted onto *Momordica* treated at  $42^\circ\text{C}$ .

Cs-42 and Mc-42 plants decreased by 15.8% and 5.1% compared with Cs-28 plants, respectively (Fig. 2A). The carotenoid content in Cs-42 was 19.3% lower than that in Cs-28, whereas the carotenoid content in Mc-42 plants increased by 4.3% compared with that in its corresponding control plants (Fig. 2B).

#### 3.2. Effects of *Mc*-rootstock on intracellular redox status

To further investigate the role of *Mc* in response to heat stress, the REL and MDA content of 4 groups of seedlings were determined to assess their membrane thermostability. The REL and MDA content were not significantly different between Cs-28 and Mc-28 plants under the optimum growth temperature (Fig. 2C and D). However, we noticed that the high temperature treatment caused a 76.3% and 47.7% increase in REL in self-grafted cucumbers and *Mc*-grafted plants, respectively, compared with Cs-28 plants. Similarly, the MDA content in Cs-42 plants increased by 32.8% in comparison to Cs-28 plants, while it only increased by 16.6% in Mc-42 plants.

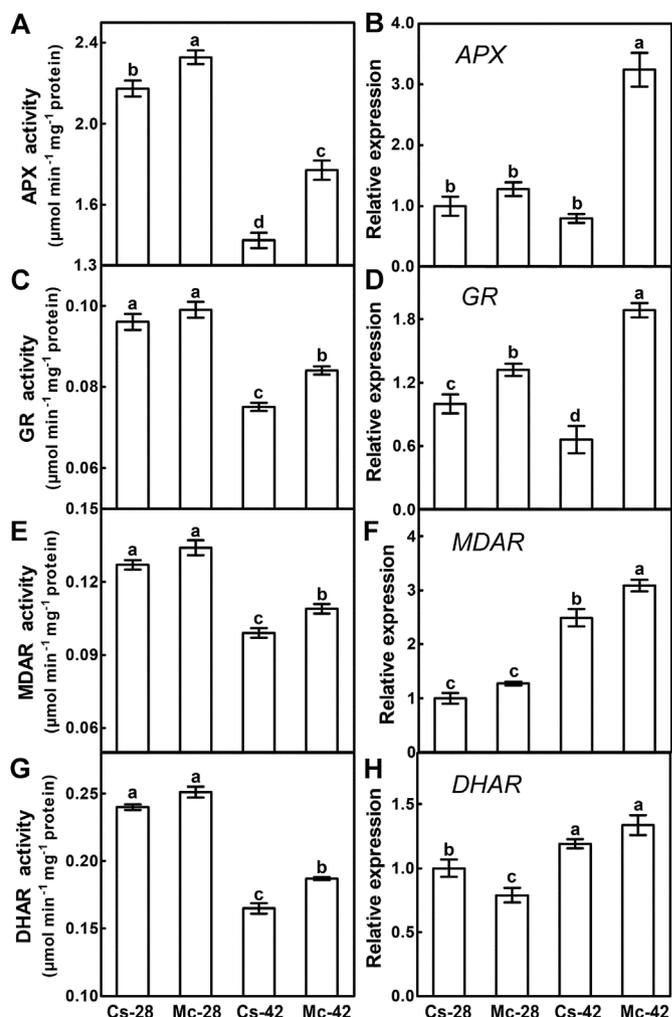
Previous studies have demonstrated that there is a close relationship between ROS over-accumulation and the disruption of photosynthesis (Muñoz et al., 2018). Considering the sensitivity of *Cs* self-grafted plants to heat stress, we analyzed the H<sub>2</sub>O<sub>2</sub> content and the O<sub>2</sub><sup>·-</sup> production rate after heat treatment for 24 h. As shown in Fig. 2E and F, there was almost no difference induced by rootstocks at  $28^\circ\text{C}$ . In contrast, the H<sub>2</sub>O<sub>2</sub> level in Cs-42 seedlings increased by 40.5% compared with that in Cs-28 plants, but it only increased by 6.4% in Mc-42 plants relative to Cs-28 plants (Fig. 2E). Although heat stress apparently enhanced the O<sub>2</sub><sup>·-</sup> production rate in self-grafted and *Mc*-grafted plants, the increase in the O<sub>2</sub><sup>·-</sup> production rate in self-grafted plants was far



**Fig. 3.** Effects of *Momordica* rootstock on the non-enzymatic antioxidant content involved in the ASA-GSH cycle in cucumbers after a 24 h-heat treatment. Values are shown as the mean  $\pm$  SE. ( $n = 3$ ). Different letters represent statistically significant differences at  $P < 0.05$  according to Duncan's multiple range tests. ASA, ascorbic acid; DHA, dehydroascorbic acid; GSH, glutathione; GSSG, oxidized glutathione; Cs-28, self-grafted cucumber seedlings treated at 28 °C; Mc-28, seedlings grafted onto *Momordica* treated at 28 °C; Cs-42, self-grafted cucumber seedlings treated at 42 °C; Mc-42, seedlings grafted onto *Momordica* treated at 42 °C.

greater than that in cucumbers grafted onto Mc after being exposed to the high temperature (Fig. 2F).

Furthermore, we evaluated the status of the ASA-GSH cycle to unveil the potential contribution of Mc rootstock in alleviating stress-induced over-accumulation of ROS. Heat stress significantly decreased the level of GSH, total glutathione and the GSH/GSSG ratio, while it had little influence on the content of DHA, GSSG, total ASA and the ASA/DHA ratio in the leaves of the Cs- and Mc-grafted plants (Fig. 3). Moreover, Mc rootstock enhanced the ASA level and the ASA/DHA ratio, as well as decreased the DHA level in comparison to Cs-grafted plants under the optimum temperature (Fig. 3A, C and G). Importantly, there was no significant difference in the content of GSH and GSSG between Mc-28 and Cs-28 plants. Nevertheless, the level of GSH in Mc-42 plants increased by 20.74% compared with that in Cs-42 plants (Fig. 3B). The enzyme assays of APX, GR, MDAR and DHAR indicated that heat stress decreased the activity of the enzymes by 34.4%, 21.9%, 22.1% and 31.3% in self-grafted plants, respectively (Fig. 4). On the other hand, Mc rootstock grafting effectively mitigated the reduction in activity of the antioxidant enzymes. The activity of APX particularly increased by 24.3% in Mc-42 plants compared with that in Cs-42 plants (Fig. 4A). In addition, Mc grafting increased the enzyme activity of GR,



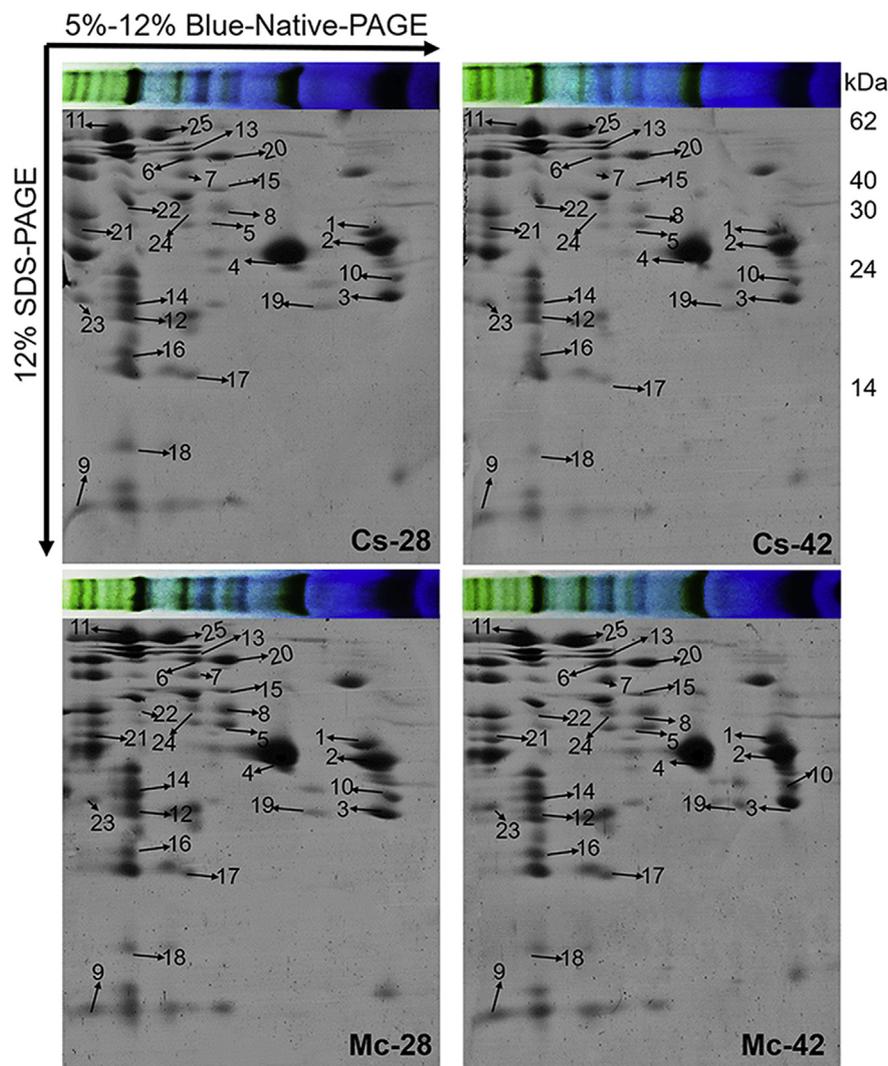
**Fig. 4.** Effects of *Momordica* rootstock on the enzymatic activity and transcription level of APX (A and B), GR (C and D), MDAR (E and F) and DHAR (G and H) of cucumbers after a 24 h heat treatment. Values are shown as the mean  $\pm$  SE. ( $n = 3$ ). Different letters represent statistically significant differences at  $P < 0.05$  according to Duncan's multiple range tests. APX, ascorbate peroxidase; GR, glutathione reductase; MDAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; Cs-28, self-grafted cucumber seedlings treated at 28 °C; Mc-28, seedlings grafted onto *Momordica* treated at 28 °C; Cs-42, self-grafted cucumber seedlings treated at 42 °C; Mc-42, seedlings grafted onto *Momordica* treated at 42 °C.

MDAR and DHAR by 12%, 10.1% and 13.3%, respectively, compared with self-grafted cucumbers after heat exposure (Fig. 4C, E and G).

Then we analyzed the transcription of APX, GR, MDAR and DHAR to test whether the Mc-induced redox modifications under the high temperature treatment occurred at the transcription level or not. Interestingly, the transcription levels of APX and MDAR were not significantly different between Cs-grafted and Mc-grafted plants under the optimum growth temperature, while the high temperature intensified the transcription of these genes in Mc-grafted cucumbers to a higher degree than that in Cs-42 plants (Fig. 4B, D and F). In particular, the expression levels of APX and GR in Mc-42 plants were approximately 3-fold and 2-fold higher than those in Cs-42 plants, respectively (Fig. 4B and D).

### 3.3. Effects of *Mc*-rootstock on thylakoid membrane proteins

To investigate the role of Mc rootstock in the response to heat stress further, optimized Blue-Native/SDS-PAGE 2-DE was applied to the entire thylakoid membrane, using 2% (w/v) n-Dodecyl-beta-D-maltoside



**Fig. 5.** Two-dimensional Blue-Native/SDS-PAGE analysis of the thylakoid membrane proteins of cucumber leaves after heat treatment for 24 h. Representative maps are shown. Proteins were stained with Coomassie Brilliant Blue (R-250). Mass spectrometry successfully identified 25 differentially expressed proteins, labeled 1–25. Detailed information of these 25 proteins is summarized in [Table 1](#). Cs-28, self-grafted cucumber seedlings treated at 28 °C; Mc-28, seedlings grafted onto *Momordica* treated at 28 °C; Cs-42, self-grafted cucumber seedlings treated at 42 °C; Mc-42, seedlings grafted onto *Momordica* treated at 42 °C.

as a gentle detergent, to characterize the Mc-induced changes of protein accumulation in cucumber leaves subjected to the high temperature. As shown on the top of the 2-DE maps ([Fig. 5](#)), diluted thylakoid membrane proteins of the grafted cucumbers were hierarchically separated into 7 major pigment–protein complexes. Significantly, there were obvious differences in protein band abundance among treatments in our experiment. In addition, at least 80 individual protein spots in each 2-DE image from the four treatments were detected after scanning the gel, and 53 of the spots on each gel were chosen for their in-depth information, including intensity, area and volume. Among the 53 protein spots, 28 spots were identified as significantly differentially accumulated with relative volume (vol. %) over 150% among treatments. Then 25 proteins, covering 21 kinds of proteins, were successfully identified by MALDI-TOF/TOF MS and matched in the NCBI *Momordica charantia*, the NCBI *Cucumis sativus* or the NCBI *Cucumis* database with a protein score confidence interval (C.I. %) over 95% ([Table 1](#)).

For a comprehensive comparison of the differential accumulation of the 21 kinds of proteins between treatments, we performed a cluster-heat map analysis using  $\log_{10}$  transformed data from Imagemaster TM 2D Platinum software ([Fig. 6A](#) and [Table S2](#)). Among the 21 proteins, 13 proteins were less abundant in the self-grafted cucumbers under the high temperature treatment. In addition, 12 of the same proteins were

more abundant in Mc-grafted cucumbers. These proteins included PsbS (spot 10), chlorophyll a-b binding protein CP29.1 (spots 1, 21), CP26 (spot 2), CP24 10A (spots 3, 23), PSII CP47 (spot 6), CP43 (spot 7), chlorophyll a-b binding protein of LHCII type 1-like (spot 4), PSI P700 apoprotein A2 (spot 11), PSI reaction center (RC) subunit II (spot 12), PSI RC subunit IV (spot 16), ATP synthase CF1 beta chain (spot 13), and Myosin-9 (spot 17). The levels of stress-induced protein KIN2-like (spot 18) and Ferredoxin-NADP reductase (spot 15) in self-grafted plants were elevated by heat shock, whereas their abundance in Mc-42 plants was lower than those in Cs-42 and Mc-28 plants.

### 3.4. Effects of Mc-rootstock on the transcriptional levels of several PSII genes

To verify the difference in the accumulation of several PSII proteins observed between treatments, we analyzed the expression of *PsbA*, *PsbB*, *PsbC*, *PsbD*, *PsbS*, *Lhcb4*, *Lhcb5* and *Lhcb6*, which encode the PSII D1, CP47, CP43, D2, PsbS, CP29, CP26 and CP24, respectively ([Figs. S1](#) and [6B](#)). Intriguingly, heat stress inhibited the expression of *PsbS*, *Lhcb4*, *Lhcb5* and *Lhcb6* in self-grafted cucumbers by 13.9%, 51.9%, 21.8% and 45.4%, respectively, while Mc-grafting stimulated the transcription of these genes after heat stress. In particular, the

**Table 1**  
Comparison of the differentially accumulated thylakoid membrane proteins among treatments based on MALDI-TOF/TOF-MS analysis.

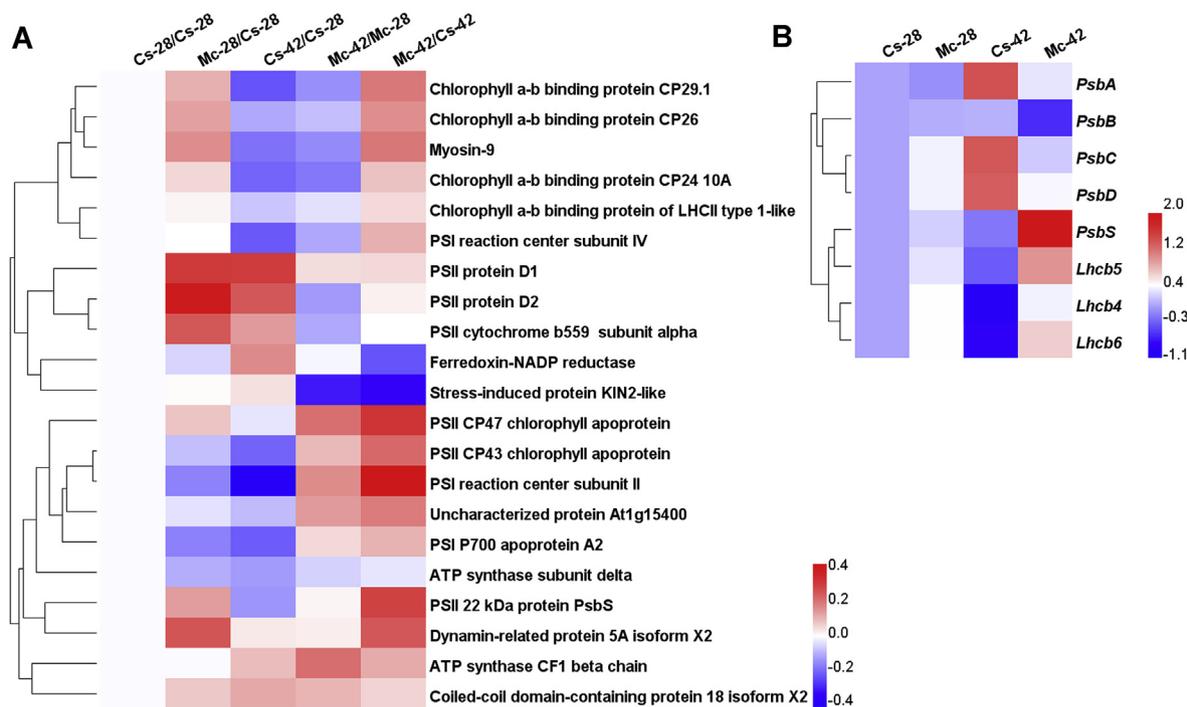
Spot No.	Protein Name	NCBI Accession No.	pI/Mr (kDa)	Unique Peptide Count	COV (%)	Protein Score C. I. %/Score	Fold changes			
							Cs-28/Cs-28	Mc-28/Cs-28	Cs-42/Cs-28	Mc-42/Mc-28
1	Chlorophyll a-b binding protein CP29.1	gi 101208740	5.67/31.08	9	33.3%	100.00/211.00	1.566	1.012	0.780	1.206
2	Chlorophyll a-b binding protein CP26	gi 101218137	5.33/30.91	7	36.7%	100.00/194.00	1.382	0.781	0.836	1.479
3	Chlorophyll a-b binding protein CP24 10A	gi 101204705	6.75/27.21	10	50.6%	100.00/142.00	1.367	0.591	0.650	1.503
4	Chlorophyll a-b binding protein of LHClI type 1-like	gi 111012353	5.29/28.19	7	16.7%	99.24/70.00	1.051	0.853	0.925	1.140
5	PSII protein D1	gi 74027082	5.12/38.95	7	22.0%	100.00/299.00	3.463	3.389	0.823	0.841
6	PSII CP47 chlorophyll apoprotein	gi 74027126	6.28/55.87	12	29.8%	100.00/182.00	1.223	0.938	1.658	2.162
7	PSII CP43 chlorophyll apoprotein	gi 74027097	6.23/50.33	8	20.8%	100.00/211.00	0.831	0.619	1.269	1.704
8	PSII protein D2	gi 74027096	5.33/39.50	5	18.5%	100.00/125.00	2.621	1.829	0.740	1.061
9	PSII cytochrome b559 subunit alpha	gi 74027118	4.83/9.38	3	37.7%	100.00/105.00	1.838	1.415	0.777	1.010
10	PSII 22 kDa protein PsbS	gi 101206477	6.77/29.04	8	34.6%	100.00/114.00	1.400	0.731	1.049	2.009
11	PSI P700 apoprotein A2	gi 74027100	6.72/82.25	10	14.5%	100.00/156.00	0.692	0.573	1.195	1.442
12	PSI reaction center subunit II	gi 101207214	9.54/22.71	10	49.4%	99.99/89.00	0.686	0.378	1.489	2.701
13	ATP synthase CF1 beta chain	gi 74027108	5.11/53.81	13	38.7%	100.00/304.00	1.001	1.255	1.674	1.335
14	ATP synthase subunit delta	gi 101211740	9.13/25.84	5	22.0%	100.00/97.00	0.791	0.744	0.882	0.937
15	Ferredoxin-NADP reductase	gi 101216095	8.54/40.50	10	34.6%	95.55/61.00	0.890	1.496	0.984	0.586
16	PSI reaction center subunit IV	gi 111014100	9.30/14.94	4	37.6%	98.41/63.00	1.009	0.596	0.775	1.312
17	Myosin-9	gi 111008282	4.77/166.30	28	23.7%	98.29/62.00	1.486	0.650	0.706	1.612
18	Stress-induced protein KIN2-like	gi 111026055	6.54/6.99	8	100.0%	96.89/60.00	1.025	1.118	0.484	0.444
19	Dynamidin-related protein 5A isoform X2	gi 111019871	8.49/66.26	17	35.0%	95.07/58.00	1.850	1.087	1.074	1.828
20	Coiled-coil domain-containing protein 18 isoform X2	gi 103488928	4.74/203.38	32	15.1%	95.09/62.00	1.209	1.346	1.294	1.163
21	Chlorophyll a-b binding protein CP29.1	gi 101208740	5.67/31.07	7	33.0%	100.00/137.00	1.083	0.198	0.633	3.458
22	PSI P700 apoprotein A2	gi 74027100	6.72/82.25	10	14.9%	100.00/144.00	0.647	0.747	0.910	0.790
23	Chlorophyll a-b binding protein CP24 10A	gi 111012163	7.93/27.57	6	37.5%	100.00/110.00	0.667	0.688	0.732	0.710
24	PSII protein D1	gi 74027082	5.12/38.95	7	22.2%	100.00/230.00	1.045	1.059	1.928	1.902
25	Uncharacterized protein At1g15400	gi 101223226	10.82/13.61	9	84.5%	97.85/66.00	0.927	0.826	1.410	1.581

<sup>a</sup> Spot numbers corresponding to spots in Fig. 5.

<sup>b</sup> Mr and pI are the molecular mass and isoelectric point, respectively.

<sup>c</sup> Percentage of sequence coverage by matched peptides.

<sup>d</sup> C. I. % is the confidence interval of protein score.



**Fig. 6.** Hierarchical cluster analysis of the differential expression of proteins (A) and photoprotection-related genes (B). Data in the clustering heat map originated from standardized vol. % of protein spots and relative abundance of genes after  $\log_{10}$  and  $\log_2$  transformation, respectively, before using HemI software. The color scale at the right indicates the relative expression levels from low (blue) to high (red). *PsbA*, the gene encoding D1 protein; *PsbB*, the gene encoding CP47 protein; *PsbC*, the gene encoding CP43 protein; *PsbD*, the gene encoding D2 protein; *PsbS*, the gene encoding PsbS protein; *Lhcb4*, the gene encoding CP29 protein; *Lhcb5*, the gene encoding CP26 protein; *Lhcb6*, the gene encoding CP24 protein; Cs-28, self-grafted cucumber seedlings treated at 28 °C; Mc-28, seedlings grafted onto Momordica treated at 28 °C; Cs-42, self-grafted cucumber seedlings treated at 42 °C; Mc-42, seedlings grafted onto Momordica treated at 42 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

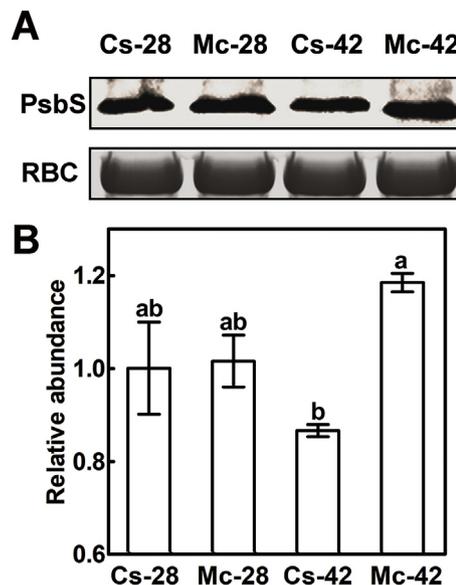
expression level of *PsbS* in Mc-42 plants was more than 4-fold as that in Cs-42 plants. There was a discrepancy between the levels of CP47 accumulation and the transcription of *PsbB* (Fig. 6). Cs-42 plants exhibited a slight higher expression level of *PsbB* compared with Cs-28 plants, but Mc-42 plants showed a lower relative abundance of *PsbB* than the control plants.

### 3.5. Validation of *PsbS* accumulation

Considering the upregulation of *PsbS* accumulation revealed by proteomic evaluation and the increase of *PsbS* abundance in Mc-42 plants, immunoblotting was conducted to verify these results (Figs. 6A and 7). Quantification of *PsbS* indicated a similar pattern to the initial proteomic evaluation. The data showed that there was only a slight difference among treatments at 28 °C. However, compared to Cs-28 plants, the relative abundance of *PsbS* increased by 18.43% in Mc-42 plants and decreased by 13.46% in Cs-42 plants (Fig. 7). Moreover, this result was consistent with the pattern in qN level among the treatments (Fig. 1E).

## 4. Discussion

The heat tolerance of plants has been extensively studied in past decades due to its crucial role in crop growth and productivity. The efficiency of photosynthesis directly affects plant yield. Therefore, the current study synthetically illustrated that Mc grafting enhanced the heat tolerance of cucumber through stabilization of the redox status and thylakoid member proteins.



**Fig. 7.** Differential accumulation of the *PsbS* protein in grafted cucumber leaves after a 24 h-heat treatment. The rubisco (RBC) protein was stained with Coomassie Brilliant Blue as the loading control (A). The quantification of *PsbS* accumulation by Image J is shown (B). Values are shown as the mean  $\pm$  SE. ( $n = 3$ ). Different letters represent statistically significant differences at  $P < 0.05$  according to Duncan's multiple range tests. Cs-28, self-grafted cucumber seedlings treated at 28 °C; Mc-28, seedlings grafted onto Momordica treated at 28 °C; Cs-42, self-grafted cucumber seedlings treated at 42 °C; Mc-42, seedlings grafted onto Momordica treated at 42 °C.

#### 4.1. *Mc-rootstock mitigated photoinhibition and mediated PsbS-involved NPQ*

In our study, destruction of the photosynthetic apparatus, reflected by a decreased Fv/Fm, and inhibition of the water splitting complex, indicated by an improved F<sub>0</sub>, were found in plants after being exposed to the high temperature (Fig. 1A, B and C). However, Mc grafting relieved the photosynthetic hypersensitivity to high temperatures. In addition, heat stress could have overburdened the photosynthetic electron transport chain and caused the deficient utilization of light energy, which was shown by the lower value of ΦPSII in Mc- and Cs-grafted plants. The higher value of ΦPSII observed in Mc-42 cucumbers compared with Cs-42 cucumbers indicated that a relatively normal photochemical energy conversion was present in heat-exposed cucumbers grafted onto Mc (Fig. 1D). More importantly, the Mc-induced change in the heat-dissipation capacity, indicated by qN, may help maintain the productivity of the photosynthetic apparatus and may help avoid the detrimental consequences of over-heating (Fig. 1E). Similar changes to photosynthesis-related factors have been reported when cucumbers were grafted onto different rootstocks (Li et al., 2016a).

Furthermore, our study revealed an upregulation of PsbS at the gene transcript and protein level in Mc-42 plants compared with Cs-42 cucumbers (Figs. 6 and 7). In agreement with previous studies, the overexpression of the *PsbS* gene in *Arabidopsis* results in a more efficient electron transfer and fluorescence quenching, and the enhanced stability of PSII-LHCII super-complexes reinforced the energy coordination and increased the resistance to photoinhibition (Dong et al., 2015). In addition, by applying chemical crosslinking and co-immunoprecipitation (Co-IP) techniques, the PSII core and LHCII are identified as the primary partners of PsbS in qE-specific conformational changes in *Arabidopsis* (Correagalvis et al., 2016). Therefore, the Mc-stimulated accumulation of PsbS could interact with other components of PSII to dissipate excessive heat and maintain the availability of the functional elements of the PSII-LHCII macrostructure. In turn, the relatively stable metabolism of photosynthesis-related proteins exhibited in Mc-grafted plants might be beneficial to repair the electron transfer chain obstructed by heat stress, as well as alleviate the over-excitation of energy and over-generation of ROS produced by the photosystem.

#### 4.2. *Mc-rootstock detoxified over-accumulated ROS in scions under high temperatures*

ROS are toxic by-products due to their oxidation potential. ROS are capable of non-reversible inactivation of proteins, indiscriminate disruption of other cell components, and they can also interfere with the highly efficient degradation of denatured proteins, the folding of newly synthesized proteins and other physiological or biochemical metabolism (Miller et al., 2010). MDA and REL are principally influenced by the ability to balance ROS metabolism under stress (Airaki et al., 2012). Data from our study illustrated that Mc-42 cucumbers had a greater membrane stability under heat compared with Cs-42 plants (Fig. 2C and D). This effect might be due in part to a better redox homeostasis inside cells of Mc-grafted plants. Furthermore, in comparison with self-grafted cucumbers under the high temperature, carotenoid accumulation was higher in Mc-42 plants to avoid photooxidation and the disposal of redundant energy (Leverenz et al., 2015), thereby obtaining the heat tolerance conferred by Mc rootstock (Fig. 2B).

Foyer et al. (2017) reported that the protection of PSII from functional damage called photoinhibition has been conflated with ROS damage and thus engendered initiatives to engineer plants with the over-expression of antioxidative enzymes to modify their stress responses. In our study, grafting onto heat-tolerant Mc diminished the production of ROS stimulated by the 42 °C heat treatment (Fig. 2E and F). This decrease could be attributed to the upregulation of genes that encode antioxidant enzymes, along with an increase in the abundance

of non-enzymatic antioxidants and enzyme activities (Figs. 3 and 4). Transcriptional up-regulation of genes encoding antioxidant enzymes is crucial to coping with the over-accumulation of ROS in plants exposed to various stresses (Zhang et al., 2015). Interestingly, our observations are in agreement with previous studies where high-temperature suppressed the transcripts of *APX* and *GR* in self-grafted cucumbers whereas stimulated the expression of these genes in plants grafted onto other stress-resistant rootstocks (Li et al., 2014b, 2016a).

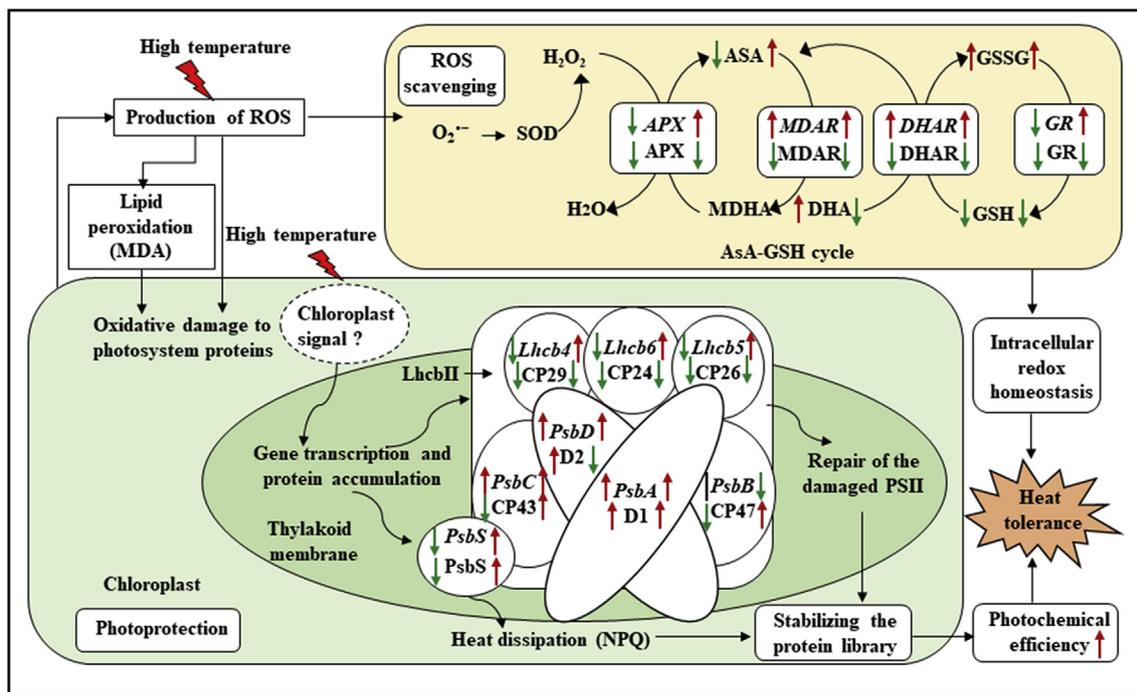
More importantly, antioxidants, such as ASA and GSH, not merely defend cellular redox-homeostasis by participating in the ASA-GSH cycle but also act as signals, which trigger safeguarding responses to resist damage (Potters et al., 2010; Foyer and Noctor, 2005). Lumen-located ASA acts as a cofactor in violaxanthin deoxidization, fulfilling the requirement of non-photochemical quenching, and even donates electrons to PSII or replaces water in the RC with inactivated oxygen evolving complex (Tóth et al., 2009). On the other hand, cellular glutathione redox homeostasis, especially the GSH/GSSG ratio, was found to affect photosynthetic efficiency (Jiang et al., 2012). Considering these findings, Mc-42 plants possessed a better state of AsA-GSH cycle, especially a higher GSH/GSSG ratio and AsA content, than Cs-42 cucumbers, which may have contributed to stabilizing redox-sensitive photosynthesis enzymes. Therefore, Mc rootstock-initiated the alleviation of photoinhibition, leading to a reduction in the over-accumulation of ROS during heat shock and allowing for a well-balanced feedback cycle.

#### 4.3. *Mc-rootstock modulated the abundance of thylakoid membrane proteins and genes transcription*

The proteomic analysis in our study indicated that the Mc rootstock stimulated the accumulation or mitigated the heat-induced degradation of several proteins, especially those PSII subunits (Fig. 6A, Fig. S1 and Table 1), and this functional response might be ascribed to the transcriptional modulation (Fig. 6B).

In *Arabidopsis*, the antisense inhibition of CP29 and CP24 lead to a macroscopic structural disruption of PSII-LHCII and severely decrease the capacity to activate NPQ in contrast to the wild type (Johnson et al., 2011). Similarly, Mc-grafting triggered the upregulation of the minor antennas, which could help to maintain the structural stabilization of the PSII-LHCII complexes. Moreover, the effective repair cycle of PSII promotes the timely degradation of damaged D1, D1 re-synthesis and the reactivation of electron transport (Gururani et al., 2015). Consequently, the Chl-a-binding proteins tightly bound to the PSII RCs around the stacked grana to compose the PSII dimer, which were found to be more highly abundant in Mc-42 than Cs-42 plants (Fig. 6A and Fig. S1). The copies of the minor antennas can be added to form PSII-LHCII macrostructures, which are more favorable to ROS detoxification and to responses to thermal-induced dissociation. Furthermore, the higher abundance of the core antenna protein CP47 in Mc-42 plants might help to prevent saturated RC from oxidation for its red-shifted spectral characteristic (D'Haene et al., 2015). After heat stress, the accumulation of D1 protein in Mc-grafted plants was higher than that in self-grafted cucumbers (Fig. 6A and Fig. S1). Hence, the orderly progression of D1 turnover might be better preserved in Mc-42 plants than Cs-42 plants, thereby accelerating the repair of the damaged PSII and playing a significant role in photoprotection under heat stress.

In addition to PSII proteins, the significantly increased accumulation of PSI RC subunit II in Mc-42 plants compared to Cs-42 plants could bind on the redox cofactors of PSI and involve in the cyclic electron flow. However, stress-induced protein KIN2-like, which was reported to be one of the cold- and ABA-induced genes in *Arabidopsis* (Kurkela and Borgfrank, 1992), was elevated after heat shock in Cs-grafted plants but decreased in Mc-grafted plants (Fig. 6A). A similar pattern was found in our study concerning the accumulation of Ferredoxin-NADP reductase (Fig. 6A); its release from the thylakoids into the stroma is essential for maintaining the NADPH/NADP<sup>+</sup> homeostasis in ROS-



**Fig. 8.** Schematic illustration of the response of self-grafted and Momordica-grafted cucumber plants to a short-term heat stress. Changes in the enzymatic activity (bottom), protein abundance (bottom), transcription level of genes (top) and the non-enzymatic antioxidant content are represented by arrows. Arrows of self-grafted and Momordica-grafted cucumber plants are presented in the left and right side, respectively. Arrow indicated up-regulation (red) and down-regulation (green). Black vertical lines represented no change. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

stressed plastids (Palatnik et al., 1997). Therefore, the lower abundance of Ferredoxin-NADP reductase in the thylakoid membrane of Mc-42 plants might be a consequence of the solubilization and transfer of Ferredoxin-NADP reductase to the stroma.

#### 4.4. Conclusion and prospective

In summary, our study provided compelling evidence that Mc rootstock triggered the alleviation of heat-induced photoinhibition in cucumber leaves. After grafting onto heat-tolerant Mc rootstocks, the protein library of the thylakoid membrane was stabilized, the production and scavenging of ROS were better balanced, and NPQ was efficiently modulated to protect the photosynthetic apparatus from inactivation caused by excess energy under heat shock (Fig. 8). However, the mechanisms for the Mc rootstock-induced heat tolerance, such as hormones, transcription factors, and the definite signaling pathway, need further study. Therefore, the emphasis of our upcoming study will be on the excavation of the molecular mechanism by which Mc rootstocks mitigate the heat stress of scions.

#### Author contributions

Ying Wei, Xinyi Wu, Sheng Shu, Jin Sun and Shirong Guo conceived and designed the experiments; Ying Wei performed the experiments and wrote the main manuscript text; Yu Wang modified the manuscript. All authors have read and approved the final manuscript.

#### Disclosures

The authors have no conflicts of interest to declare.

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#### Appendix. ASupplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.01.010>.

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