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Alterations in the porphyrin biosynthesis and antioxidant responses to chilling and heat stresses in *Oryza sativa*

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Abstract

Roles of an altered porphyrin biosynthesis and antioxidants in protection against chilling and heat stresses were evaluated in rice (*Oryza sativa* L.). When exposed to the same exposure time (6 or 30 h), heat-stressed (45 °C) plants exhibited a less oxidative stress as indicated by a lower dehydration, ion leakage, and H_2O_2 production compared to chilling-stressed (4 °C) plants. Malondialdehyde production also increased after a mild chilling stress, whereas it increased only after a long-term heat stress. The content of protoporphyrin IX, Mg-protoporphyrin IX and its methyl ester, and protochlorophyllide drastically declined under both the stresses, particularly under the long-term heat stress. Greater increases in catalase and peroxidase activities in heat-stressed plants indicate more cofactors supplied for hemoproteins compared to those of chilling-stressed and untreated control plants. Intermediates of carotenoid biosynthesis, zeaxanthin and antheraxanthin, also increased under the chilling and heat stresses. In comparison to chilling-stressed plants, heat-stressed plants were more efficient in porphyrin scavenging and antioxidant enzyme responses, which may play crucial roles in plant protection under temperature stress, thereby suffering less from oxidative stress.

Additional key words: ascorbate peroxidase, carotenoids, catalase, hydrogen peroxide, malondialdehyde, peroxidase, rice, superoxide dismutase, xanthophyll.

Introduction

Exposure of plants to low or high temperature induces many physiological changes, such as over-excitation of thylakoid membranes and a subsequent impairment of photosynthetic functions, a stunted growth, and discoloration (Berry and Björkman 1980, Guy 1990, Jung and Steffen 1997, Sanghera *et al*. 2011, Miura and Furumoto 2013). These stresses may also adversely affect photosynthetic rate, respiration rate, water relations, membrane stability, and content of phytohormones and primary and secondary metabolites (Berry and Björkman 1980, Wahid *et al*. 2007, Mittler *et al*. 2012, Qu *et al*. 2013). Although high and low temperature stresses impose different metabolic challenges, an acquired tolerance appears (Sung *et al*. 2003).

In order to cope with temperature extremes, plants implement various mechanisms including maintenance of membrane stability, scavenging reactive oxygen species (ROS), production of chaperones, and activation of transcription (Shinozaki *et al*. 2003, Yamaguchi-Shinozaki and Shinozaki 2006). During stress, disruption of cellular homeostasis is accompanied by the generation of ROS, and the extent of stress-induced damage can be attenuated by the action of cellular antioxidant systems including ascorbate, glutathione, and enzymes capable of scavenging ROS (Alscher and Hess 1993, Foyer and Shigeoka 2011, Suzuki *et al*. 2012). Superoxide dismutase (SOD) acts as the first line of defense against ROS, dismutating superoxide anion (O_2^-) to H_2O_2. Ascorbate peroxidase (APX), peroxidase (POD), and catalase (CAT) subsequently detoxify H_2O_2 (Asada 1997, Gill and Tuteja 2010, Foyer and Shigeoka 2011). Lipid-soluble antioxidants, such as carotenoids, protect membranes and are particularly important in the chloroplast (Niyogi 1999, Ledford and Niyogi 2005).

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Abbreviations: ALA - 5-aminolevulinic acid; APX - ascorbate peroxidase; CAT - catalase; DAB - 3,3-diaminobenzidine; MDA - malondialdehyde; MgProto IX - Mg-protoporphyrin IX; MgProto IX ME - Mg-protoporphyrin IX methyl ester; Pchlide - protochlorophyllide; POD - peroxidase; Proto IX - protoporphyrin IX; ROS - reactive oxygen species; RWC - relative water content; SOD - superoxide dismutase.

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Many porphyrin-containing compounds that are cofactors of apoproteins involved in photosynthesis (chlorophyll), respiration, and oxygen metabolism (heme) are extremely harmful molecules as they produce powerful radicals, such as $^1O_2$, in the presence of radiation (Duke *et al*. 1991, Wagner *et al*. 2004). The porphyrin biosynthesis starts at glutamyl-tRNA^{Glu}, and subsequently formed 5-aminolevulinic acid (ALA) is metabolized to form tetrapyrroles through a variety of reactions (Beale and Weinstein 1990). Protoporphyrin IX (Proto IX) is directed to Mg and Fe branches for chlorophyll and heme biosyntheses, respectively. Many intermediates in the porphyrin biosynthetic pathway, such as Proto IX and protochlorophyllide (Pchlide), react with O_2 to form harmful ROS (Valenzeno 1987, Reinbothe *et al*. 1996, Back and Jung 2010). The tetrapyrrole synthesis and degradation are carefully adjusted to cellular requirements reflecting different needs under varying environmental conditions (Reinbothe and Reinbothe 1996). Abiotic stresses control metabolites of the porphyrin biosynthetic pathway through scavenging tetrapyrroles in the cell, consequently attenuating photodynamic stress (Phung *et al*. 2011, Kim *et al*. 2014). Environmental factors, such as chilling or heat-stress, influence gene expression, translation, and post-translational modification of proteins involved in chloroplast biogenesis during greening process and in the dark (Tewari and Tripathy 1998, 1999, Mohanty *et al*. 2006, Abdelkader *et al*. 2007, Dutta *et al*. 2009, Dalal and Tripathy 2012, Tripathy and Dalal 2013). However, little is known about the effect of temperature stress on modulation of porphyrin metabolism in naturally-grown plants.

We attempted to follow temperature-mediated changes in tetrapyrrole biosynthesis in normally-grown plants exposed to a chilling or heat stress. We also investigated the influence of chilling and heat stresses on the carotenoid biosynthetic pathway, the accumulation of ROS, and antioxidant responses.

Materials and methods

Plant growth and stress treatment: Germinated seeds of wild-type Korean rice (*Oryza sativa* cv. Dongjin) were planted in plastic pots filled with a commercial greenhouse compost and grown for three weeks in a greenhouse at 28 to 30 °C. Three-week-old plants were transferred to a growth chamber maintained at a 14-h photoperiod, a photosynthetic photon flux density of 200 μmol m^{-2} s^{-1}, day/night temperatures of 28/25 °C and a relative humidity of 70 %. After 3 d of acclimation in the growth chamber, the plants were subjected to a chilling stress at 4 °C or a heat stress at 45 °C. The youngest fully expanded leaves were sampled after 6 h and 30 h for a mild and severe stress, respectively.

A relative water content (RWC) was determined gravimetrically in control and stressed plants. Fully expanded leaves were excised and their fresh mass (FM) was recorded immediately, and after floating on a deionized water at 4 °C overnight, their rehydrated mass (RHM) was determined. The leaves were then dried at 80 °C for 48 h to measure their dry mass (DM). RWC [%] was calculated as [(FM - DM)/(RHM - DM)] × 100 (Lee *et al*. 2005).

***In vivo* detection of H_2O_2 in plants:** According to the method of Thordal-Christensen *et al*. (1997), leaves were excised at their bases with a razor blade and a 1 mg cm^{-3} solution of 3,3-diaminobenzidine (DAB; pH 3.8) was supplied through the cut petioles for 4 h under growth chamber conditions. The experiments were terminated by the immersion of the leaves in a boiling ethanol for 10 min. This treatment decolorized the leaves except for a deep-brown polymerization product formed by the reaction of DAB with H_2O_2. After cooling, the leaves were washed at room temperature with a fresh ethanol for 4 h and photographed.

Electrolyte leakage: Electrolyte leakage was determined by a slightly modified method of King and Ludford (1983). Leaf tissue (0.2 g) was cut into small pieces and put in 15-cm^3 centrifuge tubes with 10 cm^3 of deionized water, and the conductivity of the solution was periodically measured during 4 h of gentle shaking (120 cycles per min). After the last reading, the tubes were then capped and subjected to three cycles of freezing (-20 °C) and thawing (25 °C), and final conductivity readings reflecting total conductivity were taken. The rate of conductivity was expressed as percentage of the total conductivity.

Lipid peroxidation: Lipid peroxidation was estimated according to the content of malondialdehyde (MDA) using a slight modification of the thiobarbituric acid (TBA) method described by Buege and Aust (1978). Leaf tissue (0.1 g) was homogenized with a mortar and a pestle in 5 cm^3 of a solution of 0.5 % (m/v) TBA in 20 % (m/v) trichloroacetic acid. The homogenate was centrifuged at 20 000 *g* for 15 min, and the supernatant was heated in a boiling water bath for 25 min and then cooled in an ice bath. Following centrifugation at 20 000 *g* for 15 min, the resulting supernatant was used for a spectrophotometric determination of MDA. The absorbance at 532 nm was recorded and corrected for a non-specific turbidity at 600 nm. The MDA content was calculated using a molar coefficient of absorbance of 156 mM^{-1} cm^{-1} following a formula: MDA = $[(A_{532} - A_{600})/156] \times 10^3 \times$ dilution factor.

Native PAGE analysis of antioxidant enzymes: Frozen tissue (0.5 g) was pulverized in liquid N_2 using a mortar and a pestle and then resuspended in 3 cm^3 of a 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM

ethylenediaminetetraacetic acid disodium salt, 1 % (m/v) polyvinylpyrrolidone (PVP-40), and 1 mM phenylmethylsulfonyl fluoride. The suspension was centrifuged at 15 000 *g* and 4 °C for 20 min. The supernatant was directly used for isozyme profiles assays of catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), peroxidase (POD; EC 1.11 1.7), and superoxide dismutase (SOD; EC 1.15.1.1).

Equal amounts of proteins from plants exposed to different treatments were subjected to 10 % non-denaturing polyacrylamide gels (only CAT on 7 %) at 4 °C for 2 h with a constant current of 15 mA. After completion of electrophoresis, the gels were stained for the enzymatic activities. For APX, however, 2 mM ascorbate was added to the electrode buffer, and the electrophoresis system was pre-run for 30 min before the sample was loaded. To visualize CAT isoenzymes, the gels were soaked in 3.27 mM H_2O_2 for 25 min, rinsed twice in distilled water, and stained in a freshly prepared solution containing 1 % (m/v) potassium ferricyanide and 1 % (m/v) ferric chloride (Woodbury *et al*. 1971). Isoforms of APX were visualized by incubating the gels for 30 min in a 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate. The gels were then incubated in the same buffer containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min, and then soaked in a 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM nitroblue tetrazolium (NBT) for 15 min with a gentle agitation (Rao *et al*. 1996). Staining POD isozymes was achieved by incubating the gels in a sodium citrate buffer (pH 5.0) containing 9.25 mM phenylene diamine and 3.92 mM H_2O_2 for 15 min (Olson and Varner 1993). Gels were stained for SOD isoforms by soaking them in 50 mM potassium phosphate (pH 7.8) containing 2.5 mM NBT in darkness for 25 min followed by soaking in 50 mM potassium phosphate (pH 7.8) containing 28 mM NBT and 28 µM riboflavin in darkness for 30 min. The gels were then exposed to radiation for approximately 30 min (Rao *et al*. 1996).

Porphyrin extraction and analysis: Porphyrins were extracted and analyzed following the method of Lermontova and Grimm (2000). Plant tissue (0.1 g) was grounded in 1.5 cm^3 of a medium containing methanol, acetone, and 0.1 M NaOH (9:10:1, v/v/v), and the homogenate was centrifuged at 10 000 *g* for 10 min to remove cell debris and proteins. Porphyrins were separated by HPLC using a *Novapak C18* column (4 µm of particle size, 4.6 mm × 250 mm, *Waters Chromatography*, Milford, MA, USA) at a flow rate of 1 cm^3 min^{-1}. For elution of porphyrins, a solvent system of 0.1 M ammonium phosphate (pH 5.8) and methanol (20:80) was run from 0 to 10 min followed by an 8 min linear gradient to 100 % methanol. The elution was monitored with a fluorescence detector (474, *Waters*) at excitation and emission wavelengths of 400 and 630 nm for Proto IX, and 415 and 595 nm for Mg-protoporphyrin IX (MgProto IX), MgProto IX methyl ester (ME), and Pchlide, respectively. All the porphyrins were identified and quantified using authentic standards. For chlorophyll determination, plant tissue (0.1 g) was extracted with 100 % acetone. The chlorophyll content was measured spectrophotometrically at 470, 644.8, and 661.6 nm and calculated by the method of Lichtenthaler (1987).

Carotenoid extraction and analysis: For carotenoid analysis, leaf tissue was ground in a solution of 1 cm^3 of 100 % acetone containing 10 mg of $CaCO_3$. The extract was centrifuged at 16 000 *g* for 10 min and the resulting supernatant was collected. Pigments were separated by HPLC equipped with a *Waters 2489* absorbance detector as previously described by Gilmore and Yamamoto (1991). A *Spherisorb ODS-1* column (5 µm of particle size, 4.6 mm x 250 mm) was obtained from *Alltech* (Deerfield, IL, USA). Solvent A [acetonitrile, methanol, and a 0.1 M Tris-HCl buffer (pH 8.0), 72:8:3, v/v/v] was run isocratically from 0 to 4 min followed by a 2.5 min linear gradient to 100 % solvent B (methanol and hexane, 4:1, v/v) at a flow rate of 2 cm^3 min^{-1}. A detector was set at 440 nm for the integration of peak areas.

Statistical analysis: Data were expressed as means ± SE. Differences were analyzed by the least significant differences (LSD) test. *P*-values < 0.05 were considered to be significant. The analyses were performed using the *SPSS* software (*SPSS Inc*., Chicago, USA).

Results

To compare the effect of temperature extremes on plant water status, rice plants were subjected to a chilling stress at 4 °C or a heat stress at 45 °C. Both the chilling- and heat-treated plants exhibited leaf rolling and a mild dehydration after 6 h of the treatments (Fig. 1). During the stress prolonged to 30 h, the chilling-stressed leaves were rolled and dehydrated greatly, whereas the heat-stressed leaves were dried particularly near the tips. The dehydration symptoms were correlated with RWC in the chilling- and heat-treated leaves (Fig. 2*A*). The chilling-stressed plants showed a greater decline in RWC than the heat-stressed plants indicating that the leaves of the chilling-stressed plants lost water faster than those of the heat-stressed plants.

To follow damage of cellular membranes caused by the chilling and heat stresses, we measured changes in conductivity as indication of ion leakage. The conductivity of leaf tissues gradually increased in the chilling- and heat-stressed plants with a greater increase in the chilling-stressed plants (Fig. 2*B*). The accumulations of H_2O_2 and MDA are markers of ROS production and lipid peroxidation, respectively. The plants increased the MDA content in response to the mild and severe chilling stresses, whereas the MDA content

increased only after the severe heat stress (Fig. 2*C*). The H_2O_2 production slightly increased in response to the mild chilling stress but not under the mild heat stress (Fig. 2*D*). After 30 h, the chilling-stressed plants showed a greater increase in H_2O_2 production than the heat-stressed plants.

Further, we examined effects of the chilling and heat stresses on intermediates in the porphyrin biosynthetic pathway of normally-grown green plants. Both the chilling and heat stresses decreased the content of photosensitive Proto IX already after 6 h, and it further decreased after 30 h (Fig. 3*A*). MgProto IX and MgProto IX ME also declined in response to the chilling and heat stresses and were not detected under the severe heat stress (Fig. 3*C*,*D*). The chilling-stressed plants exhibited a faster decline in Pchlide content than the heat-stressed plants although Pchlide completely disappeared under the severe heat stress (Fig. 3*E*). Unlike the decline in porphyrin intermediates in the stressed plants, the end product of the Mg-porphyrin branch, chlorophyll, slightly decreased only in response to the severe chilling and heat stresses (Fig. 3*B*).

In order to assess protective responses induced by the chilling and heat stresses, we analyzed antioxidant mechanisms. Staining activities of Cu/Zn-SOD isozyme 1 increased in response to the chilling and heat stresses

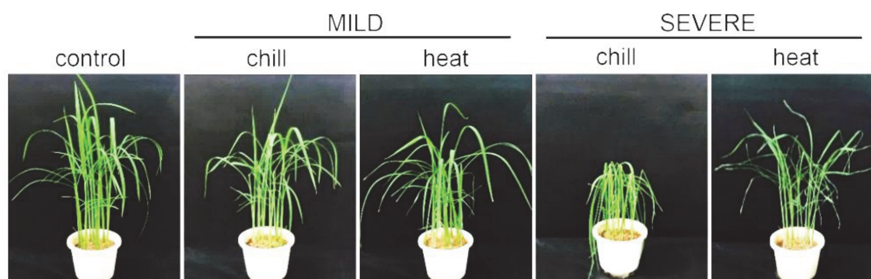

Fig. 1. Stress symptoms in rice plants exposed to chilling (4 °C) or heat (45 °C) stresses for 6 h (a mild stress) or 30 h (a severe stress). The control represents non-stressed plants.

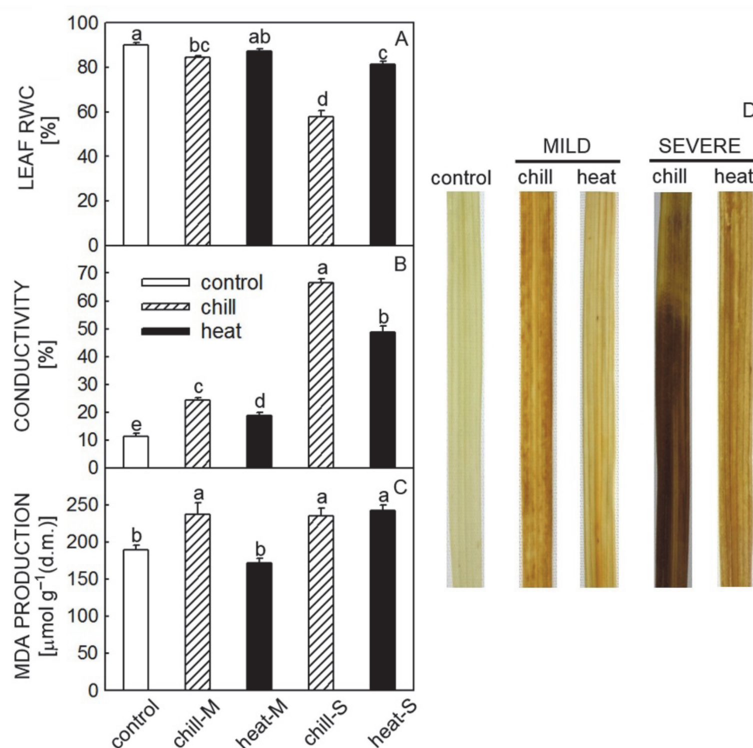

Fig. 2. Effects of chilling and heat stresses on RWC (*A*), ion leakage (conductivity; *B*), MDA production (*C*), and H_2O_2 content (DAB staining; *D*) in leaves. Brown spots represent H_2O_2 localization. Plants were subjected to the same treatments as in Fig. 1 (chill-M and heat-M represent mild chilling and heat stresses, chill-S and heat-S represent severe chilling and heat stresses, respectively). Means ± SE of six replicates from two independent experiments. Means denoted by the same letter do not differ significantly at $P < 0.05$ according to the LSD test.

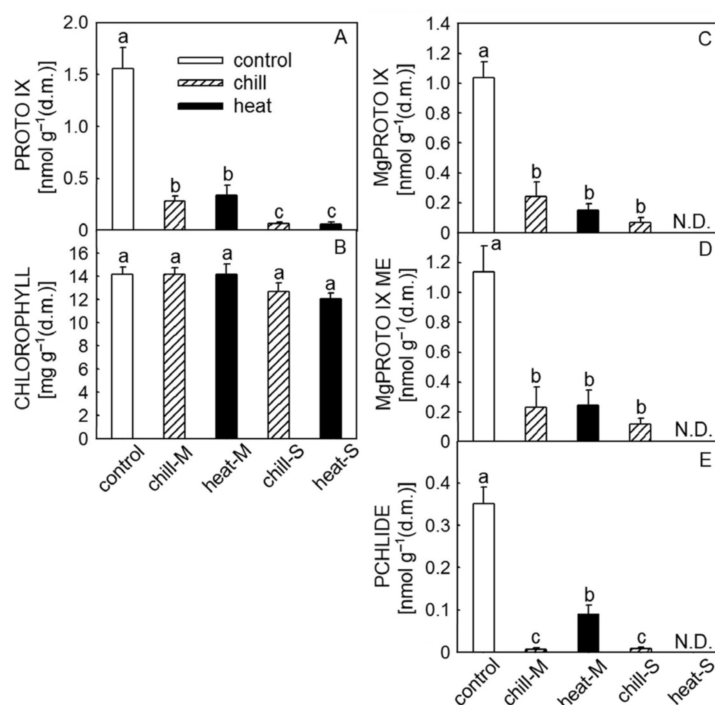

Fig. 3. The content of Proto IX (*A*), chlorophyll (*B*), and Mg-branch intermediates (*C, D, E*). Plants were subjected to the same treatments as in Fig. 1, and the treatment notations are the same as in Fig. 2. Means ± SE of six replicates from two independent experiments. Means denoted by the same letter do not differ significantly at $P < 0.05$ according to the LSD test, N.D. - not detected.

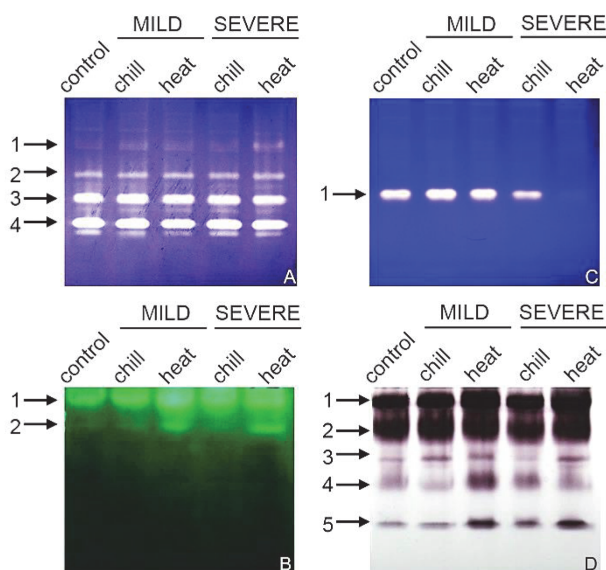

Fig. 4. Effects of chilling and heat stresses on the isozyme profiles of SOD (*A*), CAT (*B*), APX (*C*), and POD (*D*). The incubation of gels with KCN or H_2O_2 prior to staining for SOD activity suggests that SOD-2 was a Mn-SOD, whereas SOD-1, SOD-3, and SOD-4 were Cu/Zn-SOD enzymes (data not shown). Plants were subjected to the same treatments as in Fig. 1.

especially under the severe heat stress (Fig. 4*A*). The chilling- and heat-stressed plants slightly increased the activity of CAT isozyme 1 with the strongest increase under the mild heat stress, whereas CAT isozyme 2 noticeably increased only in response to the heat stress (Fig. 4*B*). Staining activities of APX increased in response to the mild chilling and heat stresses, whereas they decreased under the severe chilling stress and almost disappeared under the severe heat stress (Fig. 4*C*). In staining the activity of POD, the chilling-stressed plants increased the activities of isozymes 1 and 3 under the mild stress and isozyme 5 under the severe stress (Fig. 4*D*). The heat stress also increased the activities of POD isozymes 1, 3, and 5 with a strong increase of POD isozyme 4 in plants treated with the mild heat stress. Overall, the heat-stressed plants exhibited greater increases in the enzyme activities of SOD, CAT, and POD compared to the chilling-stressed plants.

Additionally, a role of carotenoids as non-enzymatic antioxidants was examined in plants treated with the chilling or heat stress. Noticeably, zeaxanthin was newly formed during the chilling and heat stresses with the greatest increase under the severe chilling stress (Fig. 5). Both the chilling and heat stresses markedly increased the antheraxanthin content, especially in the early stage of the stress. The content of violaxanthin gradually decreased during the chilling and heat stresses, whereas the content of neoxanthin slightly decreased only under the severe stresses (Fig. 5). Lutein and β-carotene did not change noticeably during the chilling and heat stresses.

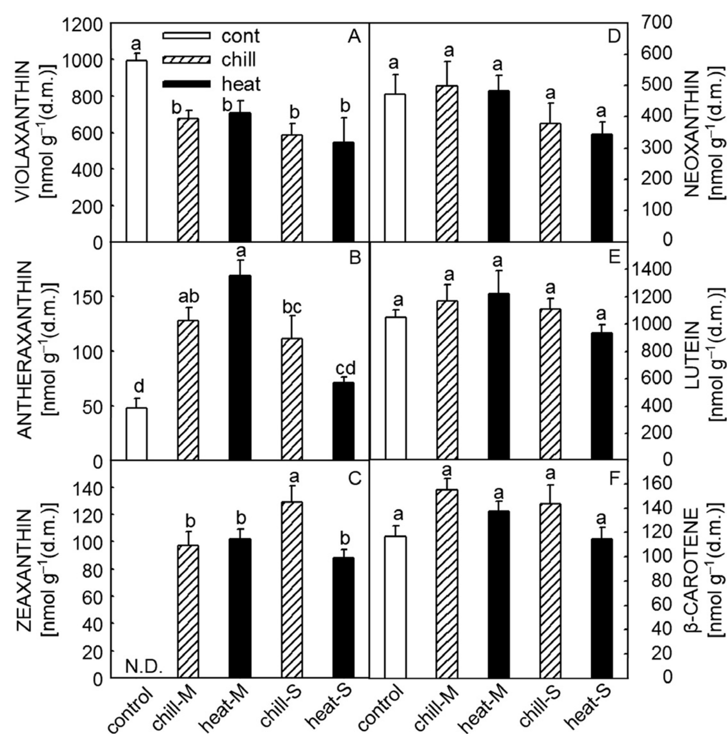

Fig. 5. Effects of chilling and heat stresses on the content of violaxanthin (*A*), antheraxanthin (*B*), zeaxanthin (*C*), neoxanthin (*D*), lutein (*E*), and β-carotene (*F*). Plants were subjected to the same treatments as in Fig. 1, and treatment notations are the same as in Fig. 2. Means ± SE of six replicates from two independent experiments. Means denoted by the same letter do not differ significantly at $P < 0.05$ according to the LSD test, N.D. - not detected.

Discussion

When plants are exposed to a low or high temperature, various adaptive, protective, and deleterious responses occur (Guy 1990, Kaplan *et al*. 2004, Sanghera *et al*. 2011, Mittler *et al*. 2012, Miura and Furumoto 2013, Qu *et al*. 2013). Our study demonstrates that both the chilling and heat stresses triggered distinct alterations in porphyrin metabolism and antioxidant systems, with a greater efficiency of porphyrin scavenging and enzymatic antioxidant responses in the heat-stressed plants.

Plant water status is an important variable under a changing ambient temperature (Mazorra *et al*. 2002). During the prolonged stress, the chilling-stressed leaves showed a greater decline in RWC compared to the heat-stressed leaves (Figs. 1, 2), indicating that the leaves of the chilling-stressed plants suffered from a greater water stress than those of the heat-stressed plants. Both the chilling- and heat-stressed plants gradually increased the electrolyte leakage and the MDA content, with greater increases in the chilling-stressed plants (Fig. 2). A primary cause of chilling injury is believed to be lipid peroxidation caused by ROS (Prasad *et al*. 1994) and the chilling-stressed plants showed a greater increase in H_2O_2 production than the heat-stressed plants. H_2O_2 is generated by the photoreduction of O_2 at photosystem I (Asada 1997) under many stress conditions, playing a central role in chloroplast-to-nucleus signaling and activating responses that range from acclimation to cell death. Chilling and heat stresses perturb the equilibrium between production and scavenging ROS in plant cells, necessitating changes in plant metabolism.

Porphyrins are other sources of ROS generation (Valenzeno 1987, Reinbothe *et al*. 1996, Phung *et al*. 2011). During high or low temperatures, chlorophyll biosynthesis and chloroplast development are perturbed in plants grown in the dark (Feierabend and Mikus 1976, Tewari and Tripathy 1998, 1999). Greening process in etiolated seedlings is also highly inhibited (Mohanty *et al*. 2006). When the green plants grown under the optimal conditions were exposed to the chilling or heat stresses, the content of Proto IX, which is mostly responsible for a rapid disruption of membranes leading to leaf death under irradiance (Tanaka and Tanaka 2006), as well as the content of MgProto IX and MgProto IX ME drastically decreased already under the mild stresses and further decreased under the severe stresses (Fig. 3). By contrast to our results, exposure to a low temperature results in the accumulation of MgProto IX in *Chlorella vulgaris* (Wilson *et al*. 2003). The activity of Mg-chelatase that converts Proto IX to MgProto IX also declines in chilling- and heat-stressed cucumber seedlings grown in the dark, whereas the activity of protoporphyrinogen IX (Protogen IX) oxidase that converts Protogen IX to

Proto IX is not substantially affected in the heat-stressed cucumber seedlings (Tewary and Tripathy 1998). In addition, the photosensitizer Pchlide decreased faster in the chilling-stressed plants than in the heat-stressed plants although it disappeared under the severe heat stress (Fig. 3*E*). Overall, the severe heat stress resulted in greater declines in Mg-porphyrin intermediates than the severe chilling stress. The drastic declines in the porphyrin intermediates during the chilling and heat stresses appear to be mainly due to porphyrin-degrading processes as well as a down-regulated porphyrin biosynthesis. The connection between down-regulation of the porphyrin biosynthesis and a stress response was shown in plants imposed to a drought stress (Phung *et al*. 2011, Dalal and Tripathy 2012). Our results also imply that the tetrapyrrole biosynthetic pathway was rapidly regulated by environmental factors that caused an imbalance in substrate flow through the pathway. The decreased content of porphyrin intermediates might function as a protective mechanism against an oxidative damage caused by the chilling and heat stresses. All living organisms must have evolved strategies to quench potentially harmful porphyrin compounds or to scavenge derived ROS. In most cases, photodestruction is prevented by the induction of protective antioxidants (Asada 1997, Niyogi 1999, Holt *et al*. 2005) as well as by a tight control of porphyrin metabolism (Reinbothe *et al*. 1996).

The steady-state content of H_2O_2, $^1O_2$, O_2^-, and hydroxyl radical depends on the balance between their generation and removal. The ROS-scavenging system includes both enzymatic and non-enzymatic constituents (Alscher and Hess 1993, Foyer and Shigeoka 2011, Suzuki *et al*. 2012). In the chilling- and heat-stressed plants, the activity of Cu/Zn-SOD isozyme 1 increased, with a greater increase under the severe heat stress (Fig. 4*A*). Cells express a set of H_2O_2-decomposing enzymes, namely CAT, APX, and glutathione peroxidase (Mittler and Poulos 2005, Gill and Tuteja 2010, Foyer and Shigeoka 2011). Particularly, plant CAT and APX are heme enzymes that function as tetrameric proteins with four Proto IX moieties (Feierabend 2005, Mittler and Poulos 2005). Staining the activities of CAT increased in response to the chilling and heat stresses with a noticeable increase of CAT isozyme 1 in the heat-stressed plants, whereas the activities of APX slightly increased in response to the mild chilling and heat stresses (Fig. 4). Increases of SOD, POD, and APX activities were also reported in a chilling-treated cucumber (Lee and Lee 2000), whereas the activities of CAT, SOD, and POD increase in response to a heat stress in wheat plants (Almeselmani *et al*. 2006). In our results, the heat-stressed plants resulted in a greater increase in the activities of CAT and POD, which indicates increased porphyrin scavenging *via* the production of hemoproteins than in the chilling-stressed plants, leading to a less oxidative damage under the heat stress. Fe-porphyrin (protoheme) is bound to various cytochromes of plastidic and mitochondrial electron transport chains and to soluble enzymes, such as CAT and POD (Beale and Weinstein 1990). By binding to a variety of different proteins, the content of free tetrapyrroles within a cell can be kept to a minimum.

Plants also respond to stress conditions by accumulating non-enzymatic antioxidants like carotenoids which protect them from harmful effects of reactive molecules including $^1O_2$ (Gilmore and Yamamoto 1993, Jung and Steffen 1997, Niyogi 1999, Jahns and Holzwarth 2012). The late steps of carotenoid biosynthesis in plants involve the conversion of violaxanthin to zeaxanthin which dissipates an excess energy as heat in an early response to an excessive radiation (Gilmore and Yamamoto 1993, Niyogi 1999). The content of antheraxanthin and zeaxanthin greatly increased during the chilling and heat stresses, whereas the content of violaxanthin decreased (Fig. 5). The elevated content of antheraxanthin and zeaxanthin, which are efficient quenchers of both triplet-state chlorophyll and $^1O_2$, appear to participate in protection against a photooxidative stress imposed by the chilling and heat stresses.

In conclusion, this study demonstrates that the porphyrin biosynthesis was modulated by environmental factors, such as the chilling and heat stresses. The heat-stressed plants were more efficient not only in the elimination of toxic porphyrin intermediates, but also in ROS scavenging by the antioxidant enzymes compared to the chilling-stressed plants, thereby leading to a less oxidative stress. Thus, it is important to understand in detail the mechanism of the modulation of porphyrin biosynthesis and photo-protective responses imposed by temperature stresses.

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