Co-Expression of Monodehydroascorbate Reductase and Dehydroascorbate Reductase from *Brassica rapa* Effectively Confers Tolerance to Freezing-Induced Oxidative Stress

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Plants are exposed to various environmental stresses and have therefore developed antioxidant enzymes and molecules to protect their cellular components against toxicity derived from reactive oxygen species (ROS). Ascorbate is a very important antioxidant molecule in plants, and monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and dehydroascorbate reductase (DHAR; EC 1.8.5.1) are essential to regeneration of ascorbate for maintenance of ROS scavenging ability. The *MDHAR* and *DHAR* genes from *Brassica rapa* were cloned, transgenic plants overexpressing either *BrMDHAR* and *BrDHAR* were established, and then, each transgenic plant was hybridized to examine the effects of co-expression of both genes conferring tolerance to freezing. Transgenic plants co-overexpressing *BrMDHAR* and *BrDHAR* showed activated expression of relative antioxidant enzymes, and enhanced levels of glutathione and phenolics under freezing condition. Then, these alteration caused by co-expression led to alleviated redox status and lipid peroxidation and consequently conferred improved tolerance against severe freezing stress compared to transgenic plants overexpressing single gene. The results of this study suggested that although each expression of *BrMDHAR* or *BrDHAR* was available to according tolerance to freezing, the simultaneous expression of two genes generated synergistic effects conferring improved tolerance more effectively even severe freezing.

INTRODUCTION

Plants grown under various environmental conditions are exposed to frequent changes in environmental conditions such as drought, chilling, freezing and salinity, which affect their growth and development, and consequently result in an increase in reactive oxygen species (Kendall and McKersie, 1989; McKersie,

1991).

Formation of reactive oxygen species (ROS) is involved in many metabolic reactions including photosynthesis, photorespiration and respiration (Baier and Dietz, 1999; Foyer and Noctor, 2000; Noctor and Foyer, 1998). Although the regular-state level of ROS is used to monitor molecules to sense their intracellular level of stress, excessive production of ROS under abnormal conditions can cause deleterious oxidations of cellular components, which result in cell death, thus the level of ROS should be tightly controlled in cellular organisms (Asada, 1987; 1999; Fridovich, 1986; Halliwell and Gutteridge, 1999; Mizuno et al., 1998).

To protect cells against the toxicity caused by ROS, plants have evolved non-enzymatic and enzymatic antioxidant mechanisms that scavenge ROS efficiently (Inze and Montagu, 1995). Ascorbate (AsA) is an essential reducing substrate for scavenging ROS (Mehlhorn et al., 1996; Nakano and Asada, 1987). APX consumes two molecules of ascorbate to detoxify H₂O₂, while generating two molecules of monodehydroascorbate (MDHA). MDHA is a radical with a short time and it is reduced back to ascorbate through monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) by using NAD(P)H as an electron donor. If reduction of MDHA does not occur rapidly, MDHA spontaneously disproportionates to ascorbate and dehydroascorbate (DHA) (Asada, 1997; Hossain and Asada, 1984a; Noctor and Foyer, 1998). DHA is reduced to AsA through dehydroascorbate reductase (DHAR; EC 1.8.5.1), which uses glutathione (GSH) as a reductant (Foyer and Halliwell, 1976). Because oxidized ascorbate (DHA) is easily hydrolyzed to 2,3-L-diketo-gulonate, instant reduction of MDHA and DHA by MDHAR and DHAR is crucial to maintenance of a proper ascorbate pool.

Monodehydroascorbate reductase is a FAD enzyme containing a thiol group in the catalytic site. MDHAR activity has been detected in several cell compartments including the chloroplast (Hossain et al., 1984), mitochondria and peroxisome,

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(Jimenez et al., 1997; Mittova et al., 2003), and cytosol (Dalton et al., 1993). MDHAR has been purified from several species including cucumber (*Cucumis astivus*) fruits (Hossain and Asada, 1985), soybean (*Glycin max*) root nodules, (Dalton et al., 1992) and potato (*Ailanthum tuberosum*) tubers (Borraccino et al., 1986). Moreover, its cDNA has been cloned from numerous species, including pea (*Pisum sativum* L.) leaves (Murthy and Zilinskas, 1994) and tomato (*Lycopersicon esculentum* Mill.) fruit (Grantz et al., 1995). MDHAR activation in response to various stress conditions has been observed in many species; namely, in *Arabidopsis* in response to high light intensity and cadmium (Leterrier et al., 2005) and UV-B radiation (Kubo et al., 1999), in tomato following exposure to salinity (Mittova et al., 2003) and high light intensity (Gechev et al., 2003), in rice subjected to low temperatures (Oidaira et al., 2000), and in Scots pine during cold accumulation (Tao et al., 1998).

DHAR is considered to be a thiol enzyme, in that thiol group(s) participate in reactions with it (Hossain and Asada, 1984b). Increments of DHAR activity have been described in response to various ROS-inducing stresses including hydrogen peroxide, methyl viologen, ozone, salt, drought, and low temperature treatments (Eltayeb et al., 2006; Lee et al., 2007; Ushimaru et al., 2006). DHAR protein has been purified and characterized from several species including spinach leaves (Hossain and Asada, 1984b), potato tubers (Dipierro and Borraccino, 1991), and rice (Kato et al., 1997). Moreover, the cDNA of the DHAR gene has been cloned from rice and its expression was induced at high temperature in rice seedlings (Urano et al., 2000).

Changes in the transcription level of the MDHAR gene in *Brassica rapa* have been observed in response to various oxidative stress conditions including H₂O₂, salicylic acid, paraquat and ozone (Yoon et al., 2004). To further understand the simultaneous effects of the gene involved in ascorbate regeneration under oxidative stress conditions, we isolated the MDHAR (*BrMDHAR*) and DHAR gene from *Brassica rapa* (*BrDHAR*) and then established transgenic *Arabidopsis* overexpressing *BrMDHAR* and *BrDHAR* under control of the SWPA2 promoter [peroxidase promoter from sweet potato, oxidative stress-inducible expression (Kim et al., 2003b)]. We then generated hybrid plants overexpressing both *BrMDHAR* and *BrDHAR* by crossing each transgenic plant as a parental line. This study allows comparison of tolerance against oxidative stress between transgenic plants co-overexpressing *BrMDHAR* and *BrDHAR* and overexpressing each protein. We analyzed the metabolic contents and changes in enzymes involved in antioxidant mechanisms in transgenic and wild-type plants, and demonstrated that overexpression of *BrMDHAR* and *BrDHAR* conferred acquired tolerance to freezing-induced oxidative stress in transgenic plants that manifested as improved redox status and increased biomass relative to wild-type plants.

MATERIALS AND METHODS

Plasmid construction and *Agrobacterium*-mediated transformation

Total RNA was extracted from *Brassica rapa* leaves using an RNeasy[®] Plant Mini Kit (Qiagen), after cDNA was synthesized using the Superscript III First-Strand Synthesis System (Invitrogen). The regions encoding the MDHAR (GenBank accession no. AY039786) and DHAR (GenBank accession no. AF536329) genes were amplified by PCR using Ex Taq[™] polymerase (Takara) with the specific primers for *BrMDHAR* and *BrDHAR* genes described in Supplementary Table I. The amplified regions encoding MDHAR and DHAR were cloned into the

pH2GW7.0 and pB2GW7.0 binary vector, respectively (Karimi et al., 2002). The pH2GW7.0 (pB2GW7.0) vector constructed naturally to control cloned genes under the CaMV 35S promoter was reconstructed to place the inserted gene under control of the oxidative stress inducible promoter, SWPA2 (oxidative stress-inducible peroxidase promoter from sweet potato). Each plasmid harboring *BrMDHAR* and *BrDHAR* under control of SWPA2 promoter was transformed into *Agrobacterium tumefaciens* GV3101 by the freeze-thaw method (Chen et al., 1994). The recombinant plasmids were then introduced into *Arabidopsis* (*Arabidopsis thaliana*, ecotype Columbia) plants using the *Agrobacterium*-mediated transformation method (Clough and Bent, 1998).

Plant growth conditions and selection of transgenic plants

The *Arabidopsis* ecotype Columbia seeds were vernalized on MS medium at 4°C for 2 days before being subjected to growth conditions (25°C, 100 µmol m⁻² s⁻¹ of light with a 16 h light, 8 h dark cycle). The 7-day-old *Arabidopsis* seedlings were then transferred into pots containing Sunshine soil Mix #5 (vermiculite = 3 : 1) and cultivated in a growth room (22°C under 100 µmol m⁻² s⁻¹ of light applied via a 16 h light, 8 h dark cycle).

Hygromycin/phosphinothricin resistance supplied by the binary vector pH2GW7.0/pB2GW7.0 was tested to select transgenic plants. Plants resistant to hygromycin/phosphinothricin were considered to be transgenic plants with the *BrMDHAR/BrDHAR* gene introduced into their genomic DNA. Fifty plants of independent transgenic lines (T1) were selected, and the generation of T3 homozygous plants was used for subsequent experiments including stress tolerance assays. More than ten independent lines that showed tolerance to oxidative stress were selected at random, and the experiments were performed using two independent transgenic lines. Transgenic plants overexpressing either *BrMDHAR* or *BrDHAR* were then crossed to establish hybridized plants overexpressing both *BrMDHAR* and *BrDHAR*. Finally, 50 plants from independent transgenic lines (T1) were selected, and T3 homozygous plants were used for subsequent experiments.

RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from the leaves using an RNeasy[®] Plant Mini Kit (Qiagen), and cDNA was synthesized with Superscript[™] III reverse transcriptase according to the manufacturer's instructions (Invitrogen). Subsequently, each gene was amplified by PCR using the gene specific primer sets shown in Supplementary Table I. The PCR amplicon of *Tubulin5* was used as a housekeeping control.

Protein extraction and Western blot analysis

For Western blot analysis, *Arabidopsis* leaves (50 mg) were harvested, frozen in liquid nitrogen, and ground to a fine powder. The protein was then extracted as described by Shultz et al. (2005), with slight modification. The extracts were quantified using BCA[™] Protein Assay Reagent (Pierce). SDS-PAGE was conducted as previously described (Harlow and Lane, 1988), with some modifications. Briefly, 30 µg of denatured proteins without heating were separated on a 12% polyacrylamide gel and transferred to a PVDF membrane (Bio-Rad). It was hybridized with antibody, and the signal intensity of each protein was visualized using ECL Western blotting detection reagent (GE Healthcare). Antibodies to *B. rapa* MDHAR and *O. sativa* DHAR were produced in rabbits hyperimmunized by proteins purified from *E. coli*. APX, GR, SOD, GPX and PRX antibodies provided by the Agresera Antibody Shop (<http://www.agresera.com>).

com/). The TUBULIN antibody for calibration of total protein expression originated from yeast (Santa Cruz).

MDHAR and DHAR activity assay

Arabidopsis leaves (50 mg) were homogenized with 1 ml of non-denaturing extraction buffer (0.1 M Tris-HCl, pH 7.5, 0.7 M sucrose, 0.1 M KCl, 10 mM DTT, 5 mM EDTA, 1 mM PMSF and protease inhibitor cocktails). The slurry was then incubated on ice for 15 min with shaking, after which it was centrifuged at $14,000 \times g$ at 4°C for 15 min. The cleared supernatants (crude extract) were used immediately for the enzyme assay.

Monodehydroascorbate reductase activity was measured spectrophotometrically using the method described by Hossain et al. (1984), with some modifications. The decrease in absorbance at 340 nm due to NADH oxidation was then monitored (absorbance coefficient $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$).

Dehydroascorbate reductase activity was assayed at room temperature by measuring the absorbance at 265 nm (Nakano and Asada, 1981). The increased absorbance due to GSH-dependent production of AsA was measured spectrophotometrically (absorbance coefficient $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$). Specific enzyme activity was calculated as nmol per mg protein. The protein concentration was quantified using Protein Dye Reagent (Bio-Rad) according to Bradford (1976).

Ascorbate and glutathione contents

Ascorbate (AsA) content was assayed using a method described by Gillespie and Ainsworth (2007). The AsA measurement was subsequently split into reduced AsA and total AsA. The absorbance was measured at 525 nm.

To measure the glutathione content in leaves, the leaves were homogenized in cold 2% metaphosphoric acid (w/v), after which the supernatant was used to measure the GSH content (Luwe et al., 1993). Total glutathione (GSH + GSSG) and oxidized glutathione (GSSG) were assayed spectrophotometrically using the specific enzyme method described by Brehe and Burch (1976), which was slightly modified. The change in absorbance at 412 nm was measured. The reduced GSH content was then calculated by subtracting the GSSG from the total GSH.

Redox state analysis

Protein was extracted from *Arabidopsis* leaves (50 mg) using the method described in the MDHAR and DHAR activity assay section. The intracellular hydrogen peroxide level was determined by ferrous ion oxidation in the presence of a ferric ion indicator, xylenol orange (Wolff, 1994). The concentration of hydrogen peroxide was measured at 560 nm.

For measurements using the oxidant-sensitive probe DCFH-DA (6-Carboxy-2',7'-dichlorofluorescein diacetate, Invitrogen), plant protein was extracted using cold-PBS buffer, after which $10 \mu\text{M}$ DCFH-DA was added to $50 \mu\text{g}$ protein. The mixture was then incubated for 20 min at 37°C in the dark. Next, DCFH-DA-loaded protein was plated in a 96-well plate and placed in a microplate reader (Victor3, PerkinElmer, Inc.). The excitation and emission were set at 485 nm and 530 nm, respectively. The fluorescence from each well was captured, and the percentage of changed fluorescence per well was calculated by the formula $[\Delta (F_{t30} - F_{t0})/F_{t0} \times 100]$, where F_{t30} = fluorescence at 30 min and F_{t0} = fluorescence at 0 min (Wang and Joseph, 1999). The relative fluorescence intensity was calculated to be 100% of the wild-type plants under normal conditions.

The level of lipid peroxidation in the leaves was measured in terms of malondialdehyde (MDA) content according to the method

described by Dhindsa et al. (1981). The absorbance was read at 532 nm, and the value for the non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using its extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ (Heath and Packer, 1968).

Chlorophyll content

For chlorophyll estimation, 20 mg of plant leaves were soaked in 1 ml of ethanol and incubated at 80°C for 20 min. The supernatant was then transferred to a new tube, and the process was repeated for the remaining pigments of leaves. Absorbance values were measured at 470 nm, 648 nm, and 664 nm using a spectrophotometer, and chlorophyll content was then calculated using the following formula: [chlorophyll a = $13.36 \times A_{664} - 5.19 \times A_{648}$, chlorophyll b = $27.43 \times A_{648} - 8.12 \times A_{664}$, carotenoid = $(1000 \times A_{470} - 2.14 \times \text{chlorophyll a} - 97.64 \times \text{chlorophyll b})/209$] (Lichtenthaler, 1987).

Antioxidant and free radical scavenging assay and determination of total phenolics

Arabidopsis leaves (50 mg) were harvested, frozen in liquid nitrogen, and ground to a fine powder. Next, 2 ml of methanol was added to the tissue powder and the samples were incubated at room temperature for 48 h in the dark, and then the supernatant was used in the following assays.

A free radical scavenging assay (ABTS assay) was performed using the method described by Re et al. (1999). The $100 \mu\text{l}$ of methanol extract was reacted with 1 ml of ABTS working solution. The absorbance was measured for 7 min.

A total antioxidant capacity assay (FRAP assay) was conducted using the method described by Benzie and Strain (1996), with some modification. The $100 \mu\text{l}$ of methanol extract was reacted with 1 ml of FRAP working solution and the products were analyzed by spectrophotometric analysis at 593 nm for 7 min.

The total phenol contents in methanol extracts were determined using the method described by Ainsworth and Gillespie (2007). Briefly, $100 \mu\text{l}$ of methanol extract was mixed well with $200 \mu\text{l}$ of 10% F-C reagent (Sigma) and $800 \mu\text{l}$ of 700 mM sodium carbonate was then added. Next, the assay tubes were incubated at room temperature for 2 h and the absorbance was measured spectrophotometrically at 765 nm.

Total flavonoids were calculated using the method described by Ordóñez et al. (2006). Briefly, $500 \mu\text{l}$ of methanol extracts were added to $500 \mu\text{l}$ 2% AlCl_3 ethanol solution. The reaction mixture was then incubated at room temperature for 1 h and the absorbance was measured spectrophotometrically at 420 nm.

Total flavonols were calculated using the method described by Kumaran and Karunakaran (2007). Briefly, $300 \mu\text{l}$ of methanol extracts were reacted with $300 \mu\text{l}$ of 2% AlCl_3 ethanol and $450 \mu\text{l}$ of 0.6 M sodium acetate solution, after which the samples were incubated at 20°C for 2 h 30 min and the absorbance was measured spectrophotometrically at 440 nm.

Stress tolerance assay

Arabidopsis seedlings cultivated in the growth room under the conditions described above for 7 days after being transferred to a pot were used for the freezing tolerance assay. The seedlings were subjected to a 16 h-freezing regime at -5°C, after which the temperature was increased sequentially to 22°C ($0.1^\circ\text{C min}^{-1}$). The plants were then returned to the growth room and observed for differences in recovery ability (tolerance to freezing) between wild-type and transgenic plants. Fresh weight was calculated as gram per independent plant excluding the roots.

Fig. 1. Schematic diagram of construction of the expression system and analysis of *BrMDHAR* and *BrDHAR* expression in transgenic plants. (A) Either *BrMDHAR* or *BrDHAR* expression were regulated under control of SWPA2 promoter. Co-expression of *BrMDHAR* and *BrDHAR* was attained by hybridization of each transgenic plant. LB, left board; Hyg, hygromycin resistance; p, promoter; ppt, phosphinothricin resistance; T35S, 35S terminator; RB, right board. (B) The levels of transcripts were detected by semi-quantitative RT-PCR with specific primer sets including *BrMDHAR* (first panel), *BrDHAR* (second panel), *AtMDHAR1* (third panel) and *AtDHAR3* (fourth panel). Tubulin (*Tub*) was used for normaliza-

tion. (C) The expression of *BrMDHAR* and *BrDHAR* was confirmed by Western blot analysis using anti-*BrMDHAR* and anti-*BrDHAR* antibody. 30 μ g of proteins extracted from Arabidopsis leaves were loaded onto 12% of acrylamide gel. Anti-Tubulin antibody was used as a house keeping control. (D) The expression of *BrMDHAR* and *BrDHAR* was quantified using the image J analysis program. Data represent the means $\pm$ SD of at least four independent experiments. WT, wild-type plants; BMR, transgenic plants overexpressing *BrMDHAR*; BDR, transgenic plants overexpressing *BrDHAR*; MxD, transgenic plants overexpressing both *BrMDHAR* and *BrDHAR*. Normal, plants grown under normal conditions for 10 days; Freezing, plants recovered for 5 days after freezing treatment.

For more severe stress treatment, the seedlings were subjected to a 22 h-freezing regime at -6°C and then observed for their ability to recover as described above.

RESULTS

Generation and screening of transgenic Arabidopsis overexpressing *BrMDHAR*, *BrDHAR* and both genes

PCR products containing the complete open reading frames (ORF) of the *BrMDHAR* and *BrDHAR* genes were cloned into the binary vectors pH2GW7.0 and pB2GW7.0, respectively, which had been modified to control the target gene by SWPA2 promoter and be inducible under oxidative stress (Fig. 1A, upper panel and middle panel). The plasmids harboring *BrMDHAR* and *BrDHAR* were then introduced into the *Arabidopsis* genome using *Agrobacterium* (GV 3101)-mediated transformation methods.

Among fifty independent transgenic lines, the transgenic *Arabidopsis* more tolerant of oxidative stress were adopted for subsequent experiments concerning stress tolerance assays. Selected transgenic plants overexpressing either *BrMDHAR* or *BrDHAR* were then hybridized (Fig. 1A, lower panel) and the stress tolerant independent lines were selected. Homozygous transgenic plants of the T3 generation were used in this study.

Expression of *BrMDHAR* and *BrDHAR* in transgenic Arabidopsis plants

Expression of the *BrMDHAR* and *BrDHAR* gene in transgenic plants was detected by semi-quantitative RT-PCR (Fig. 1B). It was assumed that *BrMDHAR* and *BrDHAR* expression was controlled by the SWPA2 promoter as a result of ROS generation, even under non-stress conditions, as suggested by Noctor and Foyer (1998). Transcripts of *BrMDHAR* were detected in transgenic plants overexpressing *BrMDHAR* and co-overexpressing *BrMDHAR* and *BrDHAR* (overexpressing MxD) (Fig.

1B, first panel). In addition, *BrDHAR* expression was detected in transgenic plants overexpressing *BrDHAR* and co-overexpressing *BrMDHAR* and *BrDHAR* (Fig. 1B, second panel). The transcription levels of *BrMDHAR* and *BrDHAR* under control of the SWPA2 promoter were increased upon stress conditions induced by freezing.

To clarify whether the expression of *BrMDHAR* and *BrDHAR* proteins were effective in transgenic plants, Western blot analysis was performed. The proteins corresponding to 47.8 kDa were detected using Anti-*BrMDHAR* antibody (Fig. 1C, upper panel). As shown in Fig. 1D, expression levels detected by *BrMDHAR* antibody in transgenic plants overexpressing *BrMDHAR* and MxD were 13%- and 42%-higher than those in wild-type plants under freezing conditions. Protein molecular mass of 28.4 kDa were detected by Anti-DHAR antibody (Fig. 1C, middle panel). The expression of *BrDHAR* protein increased in transgenic plants overexpressing *BrDHAR* and MxD upon freezing, as indicated by 21% and 40% higher expression levels relative to wild-type plants.

MDHAR and DHAR expression was also detected in wild-type plants due to the 92% homology between *BrMDHAR* and *AtMDHAR1*, and 89% similarity between *BrDHAR* and *AtDHAR3*. To clearly determine whether the increased expression of MDHAR and DHAR in each transgenic plant originated from *BrMDHAR* and *BrDHAR*, their endogenous transcription levels, which are specific to *Arabidopsis thaliana* *MDHAR1* and *DHAR3*, were examined (Fig. 1B, third and fourth panel). The transcript levels of *AtMDHAR1* and *AtDHAR3* were consistent in all plants, indicating that increased expressions of MDHAR and DHAR in transgenic plants were a result of the introduced *BrMDHAR* and *BrDHAR*. Therefore, these results demonstrated that the *BrMDHAR* and *BrDHAR* gene were effectively expressed in transgenic plants, and their expression level was greatest in transgenic plants overexpressing MxD.

Fig. 2. Enzyme activity assay of MDHAR and DHAR protein and the ratio of reduced ascorbate to oxidized ascorbate. (A) Total MDHAR enzyme activity was measured by monitoring NADH oxidation spectrophotometrically at 340 nm. (B) Total DHAR enzyme activity was measured by monitoring GSH-dependent production of AsA spectrophotometrically at 265 nm. The activity was represented as nmol per mg protein. (C) The ratio of reduced ascorbate (AsA) to oxidized ascorbate (DHA) was determined. The ascorbate and total ascorbate (AsA + DHA) contents were measured spectrophotometrically at 525 nm. DHA content was calculated by subtraction of AsA from AsA + DHA. The results show the means $\pm$ SD from four independent experiments.

Functional analysis of *Br*MDHAR and *Br*DHAR in transgenic plants

To confirm that the expression of *Br*MDHAR and *Br*DHAR in transgenic plants was functional, MDHAR and DHAR activity were measured. MDHAR activity was higher in transgenic plants than wild-type plants under freezing conditions. The MDHAR activities in transgenic plants overexpressing *Br*MDHAR, *Br*DHAR and MxD were 28%, 22% and 40% higher than wild-type plants in the presence of freezing (Fig. 2A). Even though MDHAR activity generally decreased under freezing conditions

Fig. 3. Redox status and lipid peroxidation. (A) The level of hydrogen peroxide was determined using FOX reagent. Oxidation of ferrous ion indicated by xylene orange was measured spectrophotometrically at 560 nm. (B) DCFHDA fluorescence intensity was detected using a microplate reader. The fluorescence intensities were measured at an excitation and emission of 485 nm and 530 nm, respectively. (C) Lipid peroxidation was estimated by measuring malondialdehyde (MDA) spectrophotometrically at an absorbance of 532 nm. Each value is the mean $\pm$ SD of six replicates ($P < 0.05$).

when compared to normal conditions, transgenic plants maintained a level of MDHAR activity similar to that observed when subjected to non-stress conditions, even under freezing conditions, whereas wild-type plants showed 23% decreased MDHAR activity under freezing conditions when compared to normal conditions.

Overall, DHAR activities were up-regulated in response to stress. DHAR activities under normal conditions showed no difference between wild-type and transgenic plants, whereas transgenic plants overexpressing *Br*MDHAR, *Br*DHAR and MxD showed 6% to 14% higher DHAR activity under freezing conditions than wild-type plants (Fig. 2B). Although the increases in the activities of MDHAR and DHAR were slight, the increase in transgenic plants was consistent in repeated experiments. Because six isozymes of MDHAR and five isozymes of DHAR are present in *Arabidopsis* and the MDHAR and DHAR assays were measured for all isozymes in plants, these increased values for MDHAR and DHAR in transgenic plants can

Fig. 4. Chlorophyll and carotenoid contents. Chlorophyll and carotenoid contents were measured spectrophotometrically using extracts from fresh leaf tissue. (A) Chlorophyll contents were calculated according to the formula described by Lichtenthaler. (B) Carotenoid content was measured based on the absorbance at 470 nm and calculation by the formula as mentioned above. Numbers shown are the means of values from four replicates.

be attributed to the activity of either *BtMDHAR* or *BtDHAR*, as well as the activity of endogenous isozymes in *Arabidopsis*. These assays showed that total MDHAR and DHAR activity in transgenic plants were higher than that in wild-type plants, and that transgenic plants overexpressing MxHAR showed a greater increase in MDHAR and DHAR activities than transgenic plants overexpressing either *BtMDHAR* or *BtDHAR*.

The levels of ascorbate, which is the product of MDHAR and DHAR reaction, were also measured. The ratio of reduced ascorbate (AsA) to oxidized ascorbate (DHA) was 7%, 11% and 16% higher in transgenic plants overexpressing *BtMDHAR*, *BtDHAR* and MxHAR under freezing conditions when compared to wild-type plants, respectively (Fig. 2C). The ratio of AsA to DHA in transgenic plants overexpressing MxHAR was 26%-higher than that of wild-type plants, even under normal conditions. Based on these results, the reduced ascorbate pool was maintained effectively in transgenic plants, especially in MxHAR overexpressing transgenic plants.

Redox status in transgenic plants

The level of hydrogen peroxide was examined by two methods. In one method, intracellular hydrogen peroxide levels were determined by ferrous ion oxidation with FOX reagent (Fig. 3A). Under normal conditions, all plants showed lower levels of hydrogen peroxide than those subjected to freezing conditions. Upon exposure to freezing stress, wild-type plants had approximately 2-fold higher hydrogen peroxide levels than those that were not subjected to stressful conditions, whereas transgenic plants overexpressing *BtMDHAR*, *BtDHAR* and MxHAR showed only 55%, 56% and 45% of the hydrogen peroxide levels observed in wild-type plants in freezing condition.

The levels of hydrogen peroxide were also analyzed by a flu-

Fig. 5. Antioxidant and free radical scavenging assay. (A) ABTS free radical scavenging activity was evaluated using methanol extract from plants. Decreased absorbance was measured spectrophotometrically at 734 nm. (B) Ferric reducing antioxidant power was calculated by measuring ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II), estimated spectrophotometrically at 593 nm. Each value is the mean $\pm$ SD of six replicates.

orescence-based assay using the oxidant (H_2O_2)-sensitive probe DCFHDA (Wang and Joseph, 1999). The change in fluorescence, which reflects the hydrogen peroxide concentration, was 2.2-fold greater in wild-type plants subjected to freezing conditions than in those subjected to normal conditions, whereas transgenic plants overexpressing *BtMDHAR*, *BtDHAR* and MxHAR showed slightly increased levels (Fig. 3B). The fluorescence of wild-type plants was 2.2-fold higher than that of transgenic plants overexpressing MxHAR under freezing conditions.

Next, we measured malondialdehyde (MDA) to quantify lipid peroxidation (Fig. 3C). The MDA levels of transgenic plants under freezing conditions increased by 1.3- to 1.5-fold relative to the levels in normal conditions, whereas wild-type plants showed 3.4-fold higher level. Wild-type plants showed 2.5-, 2.4- and 2.9-fold higher malondialdehyde (MDA) levels than transgenic plants overexpressing *BtMDHAR*, *BtDHAR* and MxHAR in the presence of freezing. These data indicated that expression of *BtMDHAR* and *BtDHAR* facilitated reduced lipid peroxidation via an improved redox status, and that redox homeostasis was better retained in transgenic plants overexpressing *BtMDHAR* and *BtDHAR*, especially MxHAR, when compared to wild-type plants subjected to freezing conditions.

Chlorophyll content

To examine whether overexpression of *BtMDHAR* and *BtDHAR* exerts a protective effect against inhibition of chlorophyll synthesis by ROS, we determined chlorophyll contents. Transgenic plants overexpressing *BtMDHAR*, *BtDHAR* and MxHAR had 44.2%,

Fig. 6. Glutathione and total phenolics contents. (A) The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) was reported. Total GSH and GSSG contents were determined spectrophotometrically at an absorbance 412 nm by an enzymatic reducing reaction using GR. GSH content was calculated by subtracting the GSSG content from the total GSH. (B) Total phenolics content was measured using Folin-Ciocalteu reagent (F-C reagent). The color development in a mixture of methanol extract with F-C reagent was observed spectrophotometrically at 765 nm. Flavonoid (C) and flavonol (D) content was determined spectrophotometrically at 420 nm and 440 nm, respectively. Numbers shown are the means of values from four experiments.

44.0% and 50.5% higher levels than wild-type plants in the presence of freezing, respectively (Fig. 4A). Wild-type plants showed 40% lower levels under freezing conditions than normal conditions, whereas transgenic plants showed slightly decreased levels.

Carotenoids were found to decrease in response to freezing (Fig. 4B). Transgenic plants overexpressing MxD showed only a minor decrease of 7.3%, while wild-type plants showed a 17% decrease in response to freezing when compared to normal conditions. These results indicated that expression of *BrMDHAR* and *BrDHAR* could facilitate maintenance of the capacity of photosynthesis by maintaining the contents of organic pigments against oxidative stress induced by freezing.

Antioxidant activity and total phenolic content

Proton radical scavenging is one of the most important attributes of antioxidant mechanisms (Mathew and Abraham, 2006). The levels of free radical scavenging were 16%, 14% and 18% higher in transgenic plants overexpressing *BrMDHAR*, *BrDHAR* and MxD, respectively, than in wild-type plants under freezing conditions (Fig. 5A).

Antioxidant power, which reflects the antioxidant potentials (Adedapo et al., 2008), was also measured. Transgenic plants overexpressing *BrMDHAR*, *BrDHAR* and MxD showed 17%, 12% and 19% higher abilities of reducing compared to wild-type plants under freezing conditions, respectively (Fig. 5B).

Next, the glutathione and total phenolic content were calculated. Glutathione is one of the most important antioxidant molecule involved in retention of retaining cellular redox homeostasis. The ratio of GSH to GSSG is shown in Fig. 6A. The ratio of GSH to GSSG increased greatly in all plants subjected to freezing, and specifically transgenic plants overexpressing *BrMDHAR*, *BrDHAR* and MxD showed 1.5-, 1.8- and 2.0-fold higher levels than wild-type plants under freezing conditions, respectively.

The bioactivity of phenolics, which are also very important plant constituents, is considered to be related to the scavenging of free radicals (Hatano et al., 1989; Mallavadhani et al., 2006; Shyr-Yi et al., 2005). Transgenic plants overexpressing *BrMDHAR*, *BrDHAR* and MxD showed 25%, 21% and 27% higher levels

than wild-type plants under freezing conditions, respectively (Fig. 6B). Specifically, transgenic plants overexpressing *BrMDHAR*, *BrDHAR* and MxD showed 9%, 14% and 19% higher levels in flavonoid contents (Fig. 6C) and 8%, 10% and 17% higher levels in flavonol contents (Fig. 6D). These data indicate that overexpression of *BrMDHAR* and *BrDHAR* elevated the accumulation of antioxidant metabolites, which had an indirect relationship with the introduced antioxidant enzymes.

Changes in expression of antioxidant enzymes

Based on the suggestion that antioxidant enzymes had correlations under abiotic stresses (Kingston-Smith and Foyer, 2000b), we analyzed the expressions of antioxidant enzymes involved in ROS scavenging mechanisms to determine whether alleviated redox homeostasis in transgenic plants increased in response to *BrMDHAR* and *BrDHAR* overexpression itself or via co-regulation with other antioxidant systems. Western blot analysis was performed to investigate several antioxidant enzymes (Fig. 7A). These enzymes were as follows: superoxide dismutase (SOD), glutathione peroxidase (GPX), Peroxiredoxin Q (PrxQ), ascorbate peroxidase (APX), and glutathione reductase (GR). Evident induction of antioxidant enzymes occurred in response to freezing stress in transgenic plants, especially in those overexpressing *BrMDHAR* and MxD, when compared to wild-type plants. The expression intensities of the detected antioxidant proteins under freezing conditions are shown in Fig. 7B, in which the expression level of wild-type plants under normal conditions was considered to be 100%. The expressions, particularly in SOD and GR, were highly up-regulated in transgenic plants under freezing conditions relative to wild-type plants. Their expressions (SOD/GR) in transgenic plants overexpressing *BrMDHAR*, *BrDHAR* and MxD were 1.4- /1.5-, 1.4- /1.5- and 1.8-fold/1.6-fold higher under freezing conditions, respectively, when compared to wild-type plants. Increased expression of GPX, PRX Q and APX was also observed in transgenic plants overexpressing *BrMDHAR* and MxD. The expressions of GPX, PRX Q and APX in transgenic plants overexpressing *BrMDHAR* were 1.4-, 1.2- and 1.2-fold higher and 1.2-, 1.3- and 1.2-fold higher in transgenic plants overexpressing MxD under freezing conditions when compared to wild-type plants, respect-

Fig. 7. Changes in expression of antioxidant enzymes. (A) Alteration in expression levels of antioxidant enzymes was analyzed by Western blot analysis. (B) The expression of antioxidant enzymes in the presence of freezing were quantified using the image J analysis program. Data represent the means $\pm$ SD of at least four independent experiments. SOD, superoxide dismutase; GPX, glutathione peroxidase; PrxQ, peroxiredoxin Q; APX, ascorbate peroxidase; GR, glutathione reductase.

Fig. 8. Stress tolerant phenotypes of transgenic plants. (A) The tolerance against freezing stress was assayed in transgenic and wild-type plants by phenotype analysis. The plants were challenged by freezing stress (-5°C for 16 h in the dark), and then recovery ability was observed for 12 days. (B) Fresh weights were measured by weighing independent plants excluding the roots. To confirm freezing tolerance, six independent experiments were conducted.

tively. Induction of antioxidant enzymes under freezing conditions was most effective in transgenic plants overexpressing MxD, except for the expression of GPX, which was most highly expressed in transgenic plants overexpressing *BtMDHAR*. In addition, transgenic plants overexpressing *BtMDHAR* appeared to be more highly influenced by co-regulation in antioxidant enzymes than transgenic plants overexpressing *BtDHAR*. These findings suggest that overexpression of *BtMDHAR* and

BtDHAR activated other antioxidant enzymes involved in ROS scavenging mechanisms in transgenic plants under freezing stress.

Tolerance against freezing-induced oxidative stress

The phenotype of transgenic and wild-type plants against freezing stress was observed to analyze stress tolerance derived from relative expression of antioxidant enzymes as well as ectopic

Fig. 9. Stress tolerant phenotypes and redox status against severe freezing conditions. (A) The tolerance against severe freezing stress (-6°C , for 22 h, in the dark) was assayed by phenotype analysis. The photographs show the difference in the ability to recover among transgenic plants. (B) Fresh weights of independent plants excluding the roots were measured. Plants that had recovered for 6 days after severe freezing were used for weighing. To confirm freezing tolerance, six independent experiments were conducted. (C) The hydrogen peroxide contents were measured in the extracts from plants recovered for 6 days after severe freezing. Each value is the mean $\pm$ SD of three replicates ($P < 0.05$).

expression of *BrMDHAR* and *BrDHAR*. *Arabidopsis* leaves seemed to be adversely affected by cold (sub-zero temperature), as indicated by their leaves having a wrinkled and translucent shape after incubation at -5°C . Specifically, wild-type plants were more hypersensitive to freezing stress than transgenic plants overexpressing *BrMDHAR*, *BrDHAR* and *MxD* (Fig. 8A). These findings indicated that MDHAR and DHAR play a crucial role in acquiring tolerance in response to freezing stress, although overexpression of these proteins did not seem to have any critical function under normal conditions. Phenotype analysis was strongly supported by the fresh weight (Fig. 8B). Specifically, the fresh weight of wild-type plants decreased by 78% in response to freezing stress when compared to normal, and transgenic plants overexpressing *BrMDHAR*, *BrDHAR* and *MxD* had 3.7-fold, 3.7-fold and 4.0-fold higher fresh weight, respectively, after challenge with sub-zero temperature relative to wild-type plants. These results demonstrated that ectopic overexpression of *BrMDHAR* and *BrDHAR* enhanced tolerance against freezing in transgenic plants with an increase in total biomass.

When plants were subjected to more severe stress (-6°C , 22 h in the dark), a co-expression effect was observed in transgenic plants overexpressing *MxD*. Specifically, a difference in the ability to recover from freezing was observed among transgenic plants (Figs. 9A and 9B). The fresh weight was 2.3 or 3.0-fold higher in transgenic plants overexpressing *MxD* after being subjected to severe stress conditions when compared to transgenic plants overexpressing either *BrMDHAR* or *BrDHAR*, and 9.4-fold higher than that of wild-type plants (Fig. 9C). Moreover, the hydrogen peroxide level was 1.4- or 1.5-fold higher in transgenic plants overexpressing either *BrMDHAR* or *BrDHAR* under severe freezing condition than in transgenic plants overexpressing *MxD*, and 1.9-fold higher in wild-type plants (Fig. 9D). These results suggested that simultaneous overexpression of *BrMDHAR* and *BrDHAR* conferred more effective tolerance to freezing compared to overexpression of single protein in transgenic plants.

DISCUSSION

In this study, we demonstrated that overexpression of *BrMDHAR* and *BrDHAR* conferred tolerance to transgenic plants against

freezing-induced oxidative stress based on physiological, biochemical and genetic studies.

Adoption of a suitable promoter is crucial to effective expression of a foreign gene. Even though CaMV 35S promoter has been extensively used in *Arabidopsis* plants, constant expression of a stress-inducible transcription factor is able to trigger truncation of plant growth and development by gene silencing. Thus, a practical promoter that controls target genes more precisely under certain exogenous stimuli is desired (Tang et al., 2008). The SWPA2 promoter cloned from the sweet potato (*Ipomoea batatas*) has been shown to exert its stress-inducible function in transgenic tobacco plants in response to environmental stress factors such as H_2O_2 , wounding, and UV treatment (Kim et al., 2003a; Tang et al., 2008). Moreover, induction of various antioxidant genes (choline oxidase, superoxide dismutase, ascorbate peroxidase, and nucleotide diphosphate kinase 2) under control of the SWPA2 promoter effectively facilitated improved tolerance to multiple abiotic stresses (oxidant, salt, chilling, drought, and high temperature) in several plants (potato, tall fescue, and sweet potato) (Ryu et al., 2009). In addition, successful induction of a target gene under control of the SWPA2 promoter, which conferred the stress tolerance effectively in *Arabidopsis* plants, was confirmed in our previous study (unpublished data). Taken together, these results suggest that the expression of antioxidant genes under control of the SWPA2 promoter is more appropriate than that under control of the CaMV 35S promoter in transgenic plants.

We established transgenic plants overexpressing *BrMDHAR*, *BrDHAR* and both proteins under control of the SWPA2 promoter. *BrMDHAR* overexpression, which conferred tolerance to freezing in transgenic *Arabidopsis*, was conducted in our former study (unpublished data), and overexpression of *BrDHAR* in transgenic *Arabidopsis* also showed enhanced tolerance to freezing stress corresponding to previous reports (Ushimaru et al., 2006; Wang et al., 2010; Yoshida et al., 2006). To investigate the effects of coexistence of both proteins, which were correlated with regeneration of ascorbate, transgenic plants overexpressing either *BrMDHAR* or *BrDHAR* were hybridized. Slight (Fig. 8) and obvious (Fig. 9) effects of co-expression to improved tolerance against freezing was observed in transgenic plants overexpressing *MxD* when compared to transgenic

plants overexpressing either *BrMDHAR* or *BrDHAR*.

Because MDHAR and DHAR used in this study were cloned from *Brassica rapa*, which is a subarctic plant that shows optimum growth at 18–21°C, we tested the tolerance against freezing as an abiotic factor inducing oxidative stress in transgenic *Arabidopsis*. Freezing promoted excessive ROS accumulation, which leads to serious damage in plant cells, and plants have been developed a variety of antioxidant mechanisms. Ascorbate (AsA) is a crucial antioxidant molecule in plants. MDHAR and DHAR are involved in the reduction of oxidized AsA to use it in detoxification of ROS.

The genes investigated in the present study originated from *B. rapa* belongs to the Brassicaceae family together with *A. thaliana*, and the *BrMDHAR* and *BrDHAR* genes are 92% and 89% homologous to the *AtMDHAR1* and *AtDHAR3* gene, respectively. Due to the high homology between *B. rapa* and *A. thaliana*, we thought that *B. rapa* genes would be suitable for examination of acquired tolerance to oxidative stress by ectopic overexpression of exogenous genes avoiding RNA interference.

Transgenic plants overexpressing *BrMDHAR*, *BrDHAR* and *MxD* showed increased expression of antioxidant enzymes (APX, GR, SOD, GPX and Prx Q) (Fig. 7), and higher levels of metabolites including ascorbate (Fig. 2C) and glutathione (Fig. 6A) under freezing conditions relative to wild-type plants. Relative expression of antioxidant enzymes caused by ectopic expression had been suggested by several reports (Kingston-Smith and Foyer, 2000b; Tseng et al., 2007). And then, free radical scavenging activity was maintained highly in transgenic plants (Fig. 5). This up-regulation led to improved redox status evidenced by lower levels of hydrogen peroxide and lipid peroxidation (Fig. 3) and higher levels of chlorophyll (Fig. 4). Finally, enhanced redox homeostasis conferred acquired tolerance against freezing and an increase in total biomass in transgenic plants (Figs. 8 and 9).

MDHA was primarily reduced by MDHAR, and non-reduced MDHA was converted to DHA, after which it was reduced by DHAR. Because overexpression of either *BrMDHAR* or *BrDHAR* in transgenic plants was sufficient to impart tolerance against freezing, the expression of *BrDHAR* in addition to *BrMDHAR* expression might not result in strikingly enhanced tolerance under freezing conditions (–5°C, 16 h). However, transgenic plants co-overexpressing *BrMDHAR* and *BrDHAR* showed improved antioxidant capacity and alleviated redox status consistently and repetitively upon freezing, and distinctly enhanced tolerance upon severe freezing stress (–6°C, 22 h) relative to transgenic plants overexpressing either *BrMDHAR* or *BrDHAR* (Fig. 9).

MDHAR activity was generally decreased in all plants in the presence of freezing stress when compared to normal conditions (Fig. 2A), whereas DHAR activity increased in all plants (Fig. 2B). Antioxidant enzymes showed different correlations with various oxidative stresses. Specifically, GR and APX increased in drought but SOD was not examined (Ramanjulu and Bartels, 2002), and CAT was highly sensitive to light (Karuppanapandian et al., 2006). MDHAR has also been shown to be most sensitive to low temperature among various antioxidant enzymes including SOD, CAT, APX, DHAR and GR (Mizuno et al., 1998). We thought that the decreased activity of MDHAR upon freezing might be due to the sensitivity to low temperature.

And, changes in the activities of antioxidant enzymes have been suggested in many reports. Specifically, enzyme activity in the presence of stress has been shown to be altered by the type of stress, the strength of stress and the origin of the gene examined, and enzyme activity was either increased, decre-

sed, or changed with time in response to stress (Mizuno et al., 1998; Schützendübel et al., 2001). Alteration of MDHAR activity, which increased in *Pinus sylvestris* and declined in poplar hybrids (*Populus x Canescens*) under cadmium stress, was investigated in previous studies (Schützendübel et al., 2001; 2002). The decreased MDHAR activity and increased DHAR activity in response to freezing observed in the present study agreed with these previous reports.

Transgenic plants overexpressing *BrMDHAR* and *BrDHAR* did not show greatly increased levels of ascorbate compared to wild-type plants (Fig. 2C), even though ascorbate is the end product of MDHAR and DHAR. The increase in ascorbate of less than 20% in transgenic plants when compared to wild-type plants seemed to be attributable to co-regulation of not only MDHAR, but also APX, which consumes ascorbate for detoxification of H₂O₂. These interactions among antioxidant enzymes have also been identified in maize (Kingston-Smith and Foyer, 2000a). Because both overexpression of MDHAR and DHAR and up-regulation of APX were induced in transgenic plants overexpressing *BrMDHAR*, *BrDHAR* and *MxD*, higher consumption of ascorbate through APX as well as increased regeneration of ascorbate through MDHAR and DHAR might have occurred simultaneously in transgenic plants under freezing conditions. This relationship among antioxidant enzymes seemed to result in an increase of less than 20% of ascorbate contents in transgenic plants. A corresponding study conducted by Mizuno et al. (1998) showed that, because the rate of increased APX activity was much higher than the increased activity of MDHAR in potato tubers during low temperature storage, the ascorbate content was decreased in plants even though the recycling system of ascorbate was activated by low temperature stress. Thus, these findings indicated that transgenic plants overexpressing *BrMDHAR*, *BrDHAR* and *MxD* had properly adjusted ascorbate contents by correlated up-regulation of antioxidant enzymes, especially APX, even though ascorbate recycling was more highly upregulated by *BrMDHAR* and *BrDHAR* overexpression under freezing stress conditions. Moreover, the finding that ascorbate level was slightly higher in transgenic plants overexpressing *BrDHAR* under freezing conditions than in transgenic plants overexpressing *BrMDHAR* was also supported as an influence of APX up-regulation. The expression of APX was approximately 1.2-fold higher in transgenic plants overexpressing *BrMDHAR* when compared to transgenic plants overexpressing *BrDHAR*. Furthermore, the relationship between antioxidant enzymes and antioxidant molecules was also supported by the ratio of glutathione (GSH) to oxidized glutathione (GSSG) and GPX expression (Figs. 6A and 7A). There were some differences in the ratio of GSH to GSSG in transgenic plants and wild-type plants comparing other examined antioxidant molecules in this study. Investigation of the ratio of GSH to GSSG revealed higher levels under normal conditions and lower levels under freezing conditions in transgenic plants overexpressing *BrMDHAR* when compared to other transgenic plants, while the levels of other antioxidant molecules were generally highest in transgenic plants overexpressing *MxD*. These unexpected levels were thought to be attributable to altered expressions of DHAR and GPX in transgenic plants. There was no overexpression of *BrDHAR* and down-/ up-regulated expression of GPX under normal/ freezing conditions in transgenic plants overexpressing *BrMDHAR*, and both DHAR and GPX consumed GSH as a reducing agent during detoxification of H₂O₂.

Plant organisms make an effort to maintain homeostasis in various physiological mechanisms containing that are designed

to respond to stress. The regulation of antioxidant enzymes for retention of homeostasis has been demonstrated in a previous study (Ushimaru et al., 2006). Specifically, transgenic plants overexpressing DHAR showed no difference in DHAR activity compared to wild-type plants, even though they showed stress tolerance derived from ectopic DHAR because down-regulation of endogenous DHAR by expression of ectopic DHAR occurred in transgenic plants. Considering such homeostasis, changes of 10% to 30% of transgenic plants compared to wild-type plants might be valuable to acquiring freezing tolerance supported obviously by increased biomass and lower ROS level.

Overall, in this study, we suggested that (i) ectopic expression of *BtMDHAR* and *BtDHAR* conferred tolerance to freezing-induced oxidative stress in transgenic plants as activating relative antioxidant enzymes. (ii) Simultaneous expression of *BtMDHAR* and *BtDHAR* conferred obviously enhanced tolerance against severe freezing conditions relative to overexpression of either *BtMDHAR* or *BtDHAR* in transgenic plants.

Our investigation demonstrated that *BtMDHAR* and *BtDHAR* were crucial to stress response, and their coinstantaneous expression generated synergistic effects in acquiring stress tolerance as indicated by improved antioxidant capacity and activated expression of antioxidant enzymes in transgenic plants co-overexpressing *BtMDHAR* and *BtDHAR* when compared to transgenic plants overexpressing only one of these proteins.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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