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The expression of *BrMDHAR* gene in chloroplasts and mitochondria enhances tolerance to freezing stress in *Arabidopsis thaliana*

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Abstract

In chloroplasts and mitochondria, antioxidant mechanisms include the ascorbate-glutathione cycle, and monodehydroascorbate reductase (MDHAR) is important for regeneration of ascorbate (AsA) from monodehydroascorbate (MDHA). To improve detoxification of reactive oxygen species (ROS), we established a construct of the *MDHAR* gene from *Brassica rapa* fused to the targeting signal peptides of *Pisum sativum* glutathione reductase (GR), which was controlled by a stress-inducible SWPA2 promoter, and introduced this expression system into *Arabidopsis thaliana*. Transgenic (TG) plants overexpressing *BrMDHAR* targeted to chloroplasts and mitochondria through signal peptides showed an elevated MDHAR activity and an increased ratio of AsA to dehydroascorbate (DHA) when compared to wild-type (WT) plants under a freezing stress. These led to increased photosynthetic parameters, redox homeostasis, and biomass in TG plants when compared to the WT plants. Our results suggest that the overexpression of the *BrMDHAR* gene targeted to chloroplasts and mitochondria conferred an enhanced tolerance against the freezing stress, and a stress adaptation of dual-targeted *BrMDHAR* was better than that of single *BrMDHAR*.

Additional key words: ascorbate-glutathione cycle, ascorbate regeneration, *Brassica rapa*, chlorophyll content, dual targeting, *Pisum sativum*, redox homeostasis, ROS, transgenic plants.

Introduction

All aerobic organisms exploit the reduction-oxidation (redox) reaction of oxygen to efficiently derive energy from the oxidation of substrates, even though this reaction frequently generates reactive oxygen species (ROS; Noctor and Foyer 1998). ROS, especially hydrogen peroxide, can be utilized as signal molecules in plant growth and development (Schroeder *et al.* 2001a,b, Foreman *et al.* 2003) or in responses to environmental stresses (Prasad *et al.* 1994, Wagner 1995). Despite this role, ROS are generally toxic to cells because they cause damage to cell components, such as proteins, lipids, and DNA (Fridovich 1986, Halliwell and Gutteridge 1990, Mizuno *et al.* 1998). Therefore, the production and removal of ROS must be strictly controlled.

Because ROS are produced at very high rates during electron transport in photosynthesis and respiration, even under normal conditions (Noctor and Foyer 1998, Jezek and Hlavatá 2005, Navrot *et al.* 2007)), chloroplasts and mitochondria evolve a dynamic network of antioxidant defenses to detoxify ROS including the ascorbate-glutathione cycle (Asada 1999, Sweetlove *et al.* 2002). In chloroplasts, the photosynthetic electron-transport chain generates singlet oxygen due to incomplete energy dissipation (Mittler 2002, Halliwell 2006), or superoxides resulting from leakage of electrons at photosystem I (Asada *et al.* 1987). Ascorbate (AsA) plays a major role in scavenging ROS (Njus and Kelley 1991) and donates an electron to ascorbate peroxidase (APX) during

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Abbreviations: APX - ascorbate peroxidase; AsA - ascorbate; *BrMHAR* - *Brassica rapa* monodehydroascorbate reductase gene; DCFH-DA - 6-carboxy-2',7'-dichlorofluorescin diacetate; DHA - dehydroascorbate; DHAR - dehydroascorbate reductase; GPX - glutathione peroxidase; GR - glutathione reductase; GSH - glutathione; MDA - malondialdehyde; MDHA - monodehydroascorbate; MDHAR - monodehydroascorbate reductase; Prx Q - peroxiredoxin Q; ROS - reactive oxygen species; SOD - superoxide dismutase; SWPA2 promoter - stress-inducible promoter from sweet potato ascorbate peroxidase; TBA - thiobarbituric acid; TCA - trichloroacetic acid; TG - transgenic; WT - wild type.
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H_2O_2 detoxification. In this process, AsA is oxidized to monodehydroascorbate (MDHA) which is then re-reduced to AsA by monodehydroascorbate reductase (MDHAR; Hossain *et al.* 1984). If this reduction reaction does not occur rapidly, MDHA spontaneously disproportionates into dehydroascorbate (DHA; Asada 1987) which is reduced to AsA by dehydroascorbate reductase (DHAR) using an electron from glutathione (GSH) (Foyer and Halliwell 1976). Oxidized glutathione (GSSG) in the DHAR reaction is reduced to GSH by glutathione reductase (GR) (Scrutton *et al.* 1990, Spickett *et al.* 2000). Based on these results, MDHAR is crucial for maintaining a reduced AsA pool in cells for plant antioxidant systems. The different isoforms of MDHAR exist in chloroplasts (Hossain *et al.* 1984), mitochondria, peroxisomes (Jimenez *et al.* 1997, Mittova *et al.* 2003), and cytosol (Dalton *et al.* 1993, Obara *et al.* 2002) and might be important for cell function (Puntarulo *et al.* 1991, Asada 1999, Møller 2001).

Dual targeting to both the mitochondria and plastids was first reported for glutathione reductase (GR) from *Pisum sativum* (Creissen *et al.* 1995). As many as 107 different proteins (Rokov-Plavec *et al.* 2008) have been reported to be dual-targeted to mitochondria and chloroplasts of seven different plants including *Physcomitrella patens*, *Oryza sativa*, and *Arabidopsis thaliana* (Carrie *et al.* 2009a,b, Xu *et al.* 2013). Dual targeting a protein of interest can be achieved using different mechanisms, such as ambiguous targeting signals, where a single targeting signal has the ability to target a protein to two distinct locations, or alternative transcription and translation initiations, where altered targeting signals are produced for a location-specific organelle (Carrie *et al.* 2009 a,b, Carrie and Small 2013, Xu *et al.* 2013). Dual targeting by ambiguous signals is of particular interest, because it questions how two distinct organelle import machineries can recognize these ambiguous signals and distinguish them from organelle-specific signals (Carrie *et al.* 2009 a,b). In *A. thaliana*, 72 proteins have been shown to be dual targeted; however, as many as 500 are predicted to be dual targeted because they contain ambiguous signals (Carrie *et al.* 2009 a,b, Carrie and Whelan 2013). In addition to dual targeting to mitochondria and chloroplasts, dual targeting to mitochondria and peroxisomes, mitochondria and cytoplasm, mitochondria and Golgi apparatus, mitochondria and plasma membrane, chloroplasts and cytoplasm, mitochondria and cytoplasm, chloroplasts and nucleus, chloroplasts and peroxisomes, and chloroplasts and endoplasmic reticulum have also been reported (Carrie *et al.* 2009 a,b, Carrie and Small 2013, Carrie and Whelan 2013). There is only a limited amount of information regarding the extent of dual targeting of orthologous proteins among species or organelles because the need for dual targeting remains largely unknown. Furthermore, actual proteins and associated genes that are responsible for a variety of enzyme activities in dual-targeted transgenic (TG) plants have not been systemically investigated.

Based on these facts, this study was undertaken to determine whether dual-targeted *BrMDHAR* improved a systemically acquired tolerance to a freezing stress in TG *Arabidopsis*. To accomplish this, a gene-expression system that performed dual targeting the cloned gene into chloroplasts and mitochondria through targeting sequences in pea GR was established. The expression system of *BrMDHAR* controlled by the oxidative stress-inducible promoter from sweet potato ascorbate peroxidase (SWPA2) (Kim *et al.* 2003) was introduced to *A. thaliana* through an *Agrobacterium*-mediated transformation. The overexpression of *BrMDHAR* in TG *Arabidopsis* was confirmed at the transcriptional and translational levels, after which an effect of *BrMDHAR* expression through dual targeting was investigated in the presence of a freezing stress.

Materials and methods

Total RNA was extracted from *Brassica rapa* L. leaves using an *RNeasy® Plant Mini Kit* (*Qiagen*, Hilden, Germany), and 0.15 μg of RNA was used to synthesize cDNA using the *Invitrogen Superscript III* first-strand synthesis system (*Life Technologies*, Carlsbad**,** CA, USA). The region coding the *MDHAR* gene (accession No. AY039786) was amplified by the polymerase chain reaction (PCR) using *ExTaq™* polymerase (*Takara*, Tokyo, Japan) with primers specific for *BrMDHAR* (Table 1). The dual-targeted pea GR precursor was also amplified by PCR from the published sequence (Table 1; Cressien *et al.* 1992). The amplified regions coding *BrMDHAR* and signal peptides were cloned into a pH2GW7.0 binary vector (http://gateway.psb.ugent.be/) to overexpress *BrMDHAR* and produce a precursor protein. The pH2GW7.0 vector was constructed to control cloned genes under an oxidative stress-inducible SWPA2 promoter (Karimi *et al.* 2002, Kim *et al.* 2003). A plasmid-harboring SWPA2 promoter was transformed into *Agrobacterium tumefaciens* strain GV3101 using the freeze-thaw method (Chen *et al.* 1994). The recombinant plasmids were then introduced into *A. thaliana* (ecotype Columbia) plants using the *Agrobacterium*-mediated transformation (Clough and Bent 1998).

To select TG plants, *Arabidopsis thaliana* L. ecotype Columbia seeds placed on a Murashige and Skoog (MS; *Sigma-Aldrich*, Buchs, Switzerland) medium were vernalized at 4 °C for 2 d before being subjected to normal growth conditions (a temperature of 25 °C, an irradiance of 130 μmol m^{-2} s^{-1}, a 16-h photoperiod, and a relative humidity of 60 %). The 7-d-old seedlings were transferred into pots containing *Sunshine Mix #5: vermiculate* (3:1; *Sun Gro Horiculture*, Seba Beach, Canada) and then grown in a growth room under the

same conditions. Sixty TG plants were selected based on hygromycin resistance supplied from the binary vector pH2GW7.0. Among homozygous 10-independent TG lines, two homozygous TG plants of the T_3 generation were used for subsequent experiments of stress tolerance.

To analyze a *BrMDHAR* expression at the transcriptional level, total RNA was extracted from the leaf tissues of the TG plants using a *RNeasy*® plant mini kit (*Qiagen*, Hilden, Germany), and cDNA was synthesized with *Invitrogen Superscript*™ *III* reverse transcriptase according to the manufacturer's instructions (*Life Technologies*). Subsequently, each gene was amplified by PCR using a gene-specific primer set (Table 1). PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 20 to 25 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. Each PCR product was electrophoresed onto a 1.2 % agarose gel, stained with ethidium bromide, and visualized using image analyses. The PCR amplicon of *tubulin* was used as housekeeping control.

For Western blot analyses of crude homogenates, *Arabidopsis* leaves (50 mg) were harvested, frozen in liquid nitrogen, and ground to a fine powder. The proteins were then prepared as described by Shultz *et al.* (2005) with slight modifications. Briefly, 0.6 cm^3 of a homogenization buffer containing 2 % (m/v) sodium dodecyl sulphate (SDS), 60 mM Tris, pH 6.8, 10 % (v/v) glycerol, 10 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM sodium salt of ethylendiaminetetraacetic acid (Na_2EDTA) and *Protease Inhibitor Cocktails* (*Sigma-Aldrich*, Buchs, Switzerland) were added to the tissue powder and incubated on ice for 15 min. Next, the mixtures were centrifuged at 14 000 *g* and 4 °C for 15 min, after which the cleared supernatants were precipitated by adding 0.6 cm^3 of cold 20 % (m/v) trichloroacetic acid (TCA; a final concentration of 10 %) - acetone, and a drop of β-mercaptoethanol (β-ME; a final concentration of 0.07 %, v/v), and then incubating at -25 °C for 2 h. The resulting protein pellet was washed 7 times with 1 cm^3 of a mixture of cold acetone and β-ME (0.07 %). Next, the pellet from the final wash step was vacuum-dried for 15 min, after which it was resuspended in the same homogenization buffer and shaken for 30 min. The samples were then centrifuged at 14 000 *g* and 25 °C for 20 min, after which the protein in the supernatant was quantified using a *BCA*™ protein assay reagent (*Pierce*, Rockford, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was subsequently conducted using the method of Harlow and Lane (1988) with some modifications. Briefly, 30 µg of denatured proteins were separated on a 12 % (m/v) polyacrylamide gel without heating, and then transferred to an *Immun-Blot*® *PVDF* membrane (*Bio-Rad*, Hercules, USA). The membranes were then incubated with a blocking solution [5 % (m/v) non-fat skim milk in 10 mM Tris-HCl (pH 7.6)-buffered saline, and 0.05 % (v/v) *Tween 20* (TBST) plus 0.02 % (m/v) sodium azide] at room temperature for 1.5 h, after which they were incubated at 4 °C overnight with each primary antibody properly diluted in the blocking solution. The blots were subsequently washed 4 times with TBST for 40 min (10 min each) and incubated for 1.5 h at room temperature with the secondary antibody. After four washes with TBST, the signal intensity of each protein was visualized using an *Amersham ECL* Western blotting detection reagent (*GE Healthcares*, Little Chalfont, Buckinghamshire, UK). Antibodies to *B. rapa* MDHAR and rice DHAR were produced in rabbits by proteins purified from *Escherichia coli* BL21 (DE3). Ascorbate peroxidase (APX), GR, superoxide dismutase (SOD), glutathione peroxidase (GPX), and peroxiredoxin Q (Prx Q) antibodies were purchased from *Agrisera AB* (*Agrisera Antibobies*, Vännäs, Sweden). The tubulin antibody (*Santa Cruz Biotechnology*, Santa Cruz, USA) from yeast was used for the calibration of total protein expression.

Chloroplasts and mitochondria were isolated using the *Percoll*® (*Sigma-Aldrich*, Buchs, Switzerland) density gradient as described previously (Eubel *et al.* 2006) with the following modifications. Differential centrifugations (1 000 *g*, 5 min/18 000 *g*, 15 min) were repeated 3 times to purify the chloroplasts and mitochondria, and in the final step, a pellet enriched with chloroplasts and mitochondria was obtained by high-speed centrifugation (18 000 *g*, 15 min). The 3-step *Percoll* gradients for density gradient centrifugation contained 18, 29, and 45 % (m/v) *Percoll* diluted with phosphate-buffered saline (PBS). The chloroplast fraction was isolated from the 18/29 % interphase by gradient centrifugation (35 000 *g*, 45 min), and the mitochondria fraction was isolated from the 29/45 % interphase.

To measure an MDHAR activity**,** leaves (50 mg) were frozen in liquid nitrogen, ground to a powder, and homogenized with 1 cm^3 of a non-denaturing homogenization buffer containing 50 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 1 mM Na_2EDTA, 1 mM PMSF, and protease inhibitor cocktails. The slurry was incubated on ice, shaken, and centrifuged at 14 000 *g* and 4 °C for 15 min. The cleared supernatants (crude homogenates) were used immediately for the enzyme assay. The MDHAR activity was measured spectrophotometrically using a method described by Hossain *et al.* (1984) with some modifications. The assay was conducted at room temperature with 1 cm^3 of a reaction mixture containing 50 mM potassium phosphate, pH 7.6, 0.1 mM NADH, 2.5 mM AsA, 0.5 unit of ascorbate oxidase (*Sigma-Aldrich*, Buchs, Switzerland), and 50 µg of the crude homogenate. The decrease in absorbance at 340 nm due to NADH oxidation was monitored. The activity was calculated using an absorbance coefficient of 6.2 mM^{-1} cm^{-1}. One unit is the amount of the enzyme that oxidizes 1 nmol of NADH per min at 25 °C. The protein content was quantified using a protein dye reagent (*Bio-Rad*, Hercules, USA).

An AsA content was assayed using the method described by Gillespie and Ainsworth (2007). Briefly,

leaves (50 mg) were homogenized in liquid nitrogen and 2 cm^3 of 6 % (m/v) TCA was added. The homogenate was then centrifuged at 10 000 *g* and 4 °C for 10 min and the supernatant was used for the assay. For total AsA, 0.2 cm^3 of the homogenate was added to 0.1 cm^3 of a 75 mM potassium phosphate buffer, pH 7.0, and 0.1 cm^3 of 10 mM DTT, and then incubated at room temperature for 10 min, after which 0.1 cm^3 of N-ethylmaleimide was added. For DHA, 0.2 cm^3 of the homogenate was added to 0.1 cm^3 of a 75 mM potassium phosphate buffer, pH 7.0, and its total volume was adjusted by adding 0.2 cm^3 of water. Both above mentioned mixtures were added to 0.5 cm^3 of 10 % TCA, 0.4 cm^3 of phosphoric acid, 0.4 cm^3 of α-bipyridyl, and 0.2 cm^3 of $FeCl_3$, and incubated at 37 °C for 1 h. The absorbance was then measured at 525 nm.

An intracellular H_2O_2 content was determined in the protein homogenate by ferrous ion oxidation in the presence of a ferric ion indicator, xylenol orange (Wolff 1994). The protein homogenate (0.05 cm^3) was added to 1 cm^3 of a ferrous oxidation-xylenol orange (FOX) reagent containing 0.1 mM xylenol orange, 0.25 mM ammonium ferrous sulfate, 100 mM sorbitol, and 25 mM sulfuric acid. The mixture was then incubated at room temperature for at least 30 min, after which it was centrifuged to remove any flocculated material before measuring the absorbance at 560 nm. For H_2O_2 measurements using the oxidant-sensitive probe, 6-carboxy-2',7'-dichlorofluorescin diacetate (DCFHDA; *Invitrogen*, San Diego, USA), plant proteins were prepared using a cold-PBS buffer, after which 10 μM DCFHDA was added to 50 μg of proteins. The mixture was then incubated in the dark at 37 °C for 20 min. The DCFHDA-loaded proteins were distributed into a 96-well plate which was placed in a *VICTOR*™ X*3* multilabel plate reader (*PerkinElmer*, Waltham, USA). The excitation and emission filters 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 as $(Ft_{30} - Ft_0)/Ft_0 \times 100$, where Ft_{30} is fluorescence at 30 min and Ft_0 is fluorescence at 0 min (Wang and Joseph 1999). The fluorescence of the untreated WT plants was considered to be 100 %. In addition, the intracellular ROS were measured in protoplasts extracted from the leaves using the method described by Yoo *et al.* (2007). The protoplasts were incubated with 2 mM H_2O_2 at room temperature for 1 h and subsequently with DCFH-DA at a final concentration of 5 μM and then were shaken for 30 min. After that they were washed with PBS, the fluorescence induced by oxidation of DCFH caused by ROS was observed microscopically with an excitation and emission at 488 and 530 nm, respectively. This method was modified from that described by Moon *et al.* (2003).

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content according to the method of Dhindsa *et al.* (1981). Briefly, 50 mg of a leaf sample was homogenized in 1 cm^3 of 0.1 % TCA and then centrifuged at 10 000 g and 4 °C for 10 min. Next, 0.25 cm^3 of the supernatant was mixed with 1 cm^3 of 20 % TCA containing 0.5 % (m/v) thiobarbituric acid (TBA). The mixture was then incubated at 95 °C for 30 min, cooled on ice for 5 min, and centrifuged at 10 000 *g* and 25 °C for 10 min. The absorbance was then read at 532 nm, from which the value for the non-specific absorption at 600 nm was subtracted. The MDA content was calculated using a coefficient of absorbance of 155 mM^{-1} cm^{-1} (Heath and Packer 1968).

For a chlorophyll content determination, 20 mg of plant leaves were soaked in 1 cm^3 of ethanol and incubated at 80 °C for 20 min. The cleared supernatant following centrifugation at 8 000 *g* and 25 °C for 10 min was then transferred to a new tube. Absorbances at 470, 648, and 664 nm were measured using a spectrophotometer *UV-260* (*Shimadzu*, Tokyo, Japan) and the chlorophyll content was calculated according to Lichtenthaler (1987).

For a stress tolerance assay, *Arabidopsis* seedlings cultivated in the growth room at above mentioned conditions for 7 d after being transferred to a pot were used. The seedlings were subjected to 16-h-freezing at -5 °C, after which temperature was gradually increased (0.5 °C min^{-1}) to 22 °C and the seedlings were grown for 8 d. The recovery capacities of the TG and WT plants were observed over time.

Significant differences ($P < 0.05$) between transgenic and WT rice plants were identified by *Origin Pro 8.0.* All the experiments were carried out at least in triplicate and the results are expressed as means and standard deviations (SD). The data are presented relative to the WT plants, which were defined as 100 %. The results for the phenotypes are means representative of two independent TG plants under identical conditions.

Table 1. Oligonucleotide sequences used in this study. The first four primers were used for the construction of an expression system, the following three primers for the semi-quantitative RT-PCR.

Primer name	Forward primer	Reverse primer
BrMDHAR	5′-CGGGTACCAAATGGCGGAGAAG-3'	5′-TCAGATCTTAGCAGCAAATGAG-3′
BrMDHAR (for targeting)	5′-CACCATGGCGGAGAAGAGCTT-3'	5′-ACGTGCAAGCACATTGCTTAACAT-3′
Transit peptide	5′AGCGGCCGCATGAACCAAGCAATGGCT-3'	5′ACCATGGACTGGCGGGCGGGGTCGGCG-3′
SWPA2	5′GAGCTCCCATGATCAGATCGATAATA-3'	5′-ACTAGTCTATCGTTCGTAAATGGTGA-3′
BrMDHAR	5′-TAGTCAAGGAGTTAAACCAGGG-3′	5′-TGTCGGCTTCTAGCGACCTT-3′
MDHAR	5′-CGGCTAGACTCCCAGGTTTC-3′	5′-ATAGAACGCAGCAATGTCGG-3′
Tubulin	5′-AGACTGGAGCTGGGAAGCAT-3′	5′-CAAGAAGGGAATGCGTTGAA-3′

Results

Complete cDNA sequences of *MDHAR* from *B. rapa* were fused to transit peptides of pea glutathione reductase, and this construct was cloned into binary vector pH2GW7.0 that had been modified to regulate the target gene under the control of the SWPA2 promoter (Fig. 1*A*). The plasmid construct containing the target gene expressed in chloroplasts and mitochondria was introduced into the *Arabidopsis* genome using *Agrobacterium*-mediated transformation methods. Two homozygous TG plants showing tolerance to oxidative stress were used in this study. The TG plants (TMR 37-3 and BMR 2-2) overexpressing *BrMDHAR* in cytosol and having tolerance to freezing were also examined (Fig. 1*A*). These combined analyses enabled an evaluation of the efficiency of targeting the expression of *BrMDHAR* to chloroplasts and mitochondria with respect to ROS scavenging. The expression of *BrMDHAR* in the TG plants was established by semi-quantitative reverse transcription-PCR (RT-PCR) (Fig. 1*B*). The amplification of *BrMDHAR* transcripts was not detected in the WT

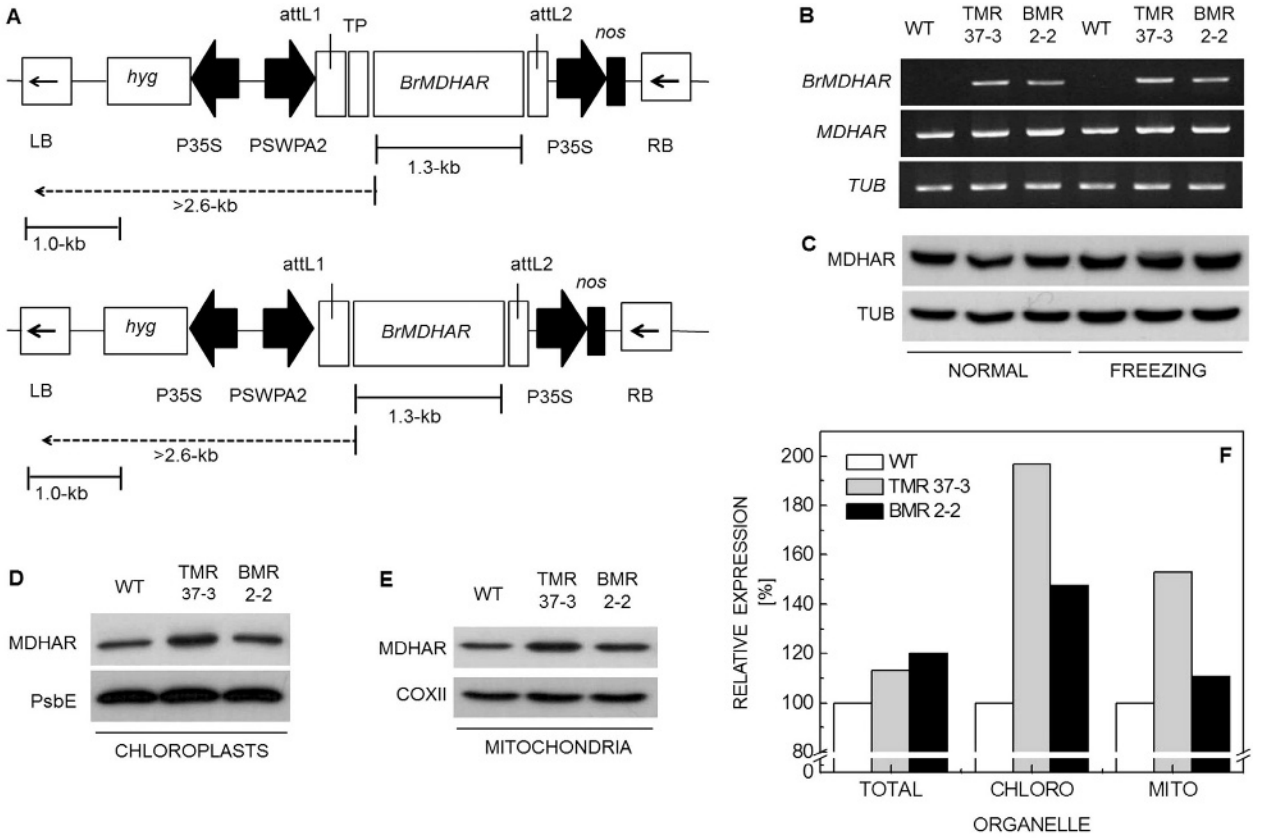

Fig. 1. The schematic diagram of the *BrMDHAR* construct targeted to chloroplasts and mitochondria, and the analysis of *BrMDHAR* expression in transgenic *Arabidopsis*. *A* - *BrMDHAR* was expressed targeted to chloroplasts and mitochondria under the control of the SWPA2 promoter (the *upper panel*). Transit peptides of pea glutathione reductase (GR) were used for the targeted expression. The *BrMDHAR* expression without a targeting signal (cytosolic expression) was also examined (the *lower panel*). *hyg* - hygromycin resistance gene, attL - DNA recombination site, LB - left border, RB - right border, TP - transit peptides of pea glutathione reductase, PSWPA2 - oxidative stress-inducible peroxidase promoter from *Ipomoea batatas*, *BrMDHAR* - *Brassica rapa* monodehydroascorbate reductase gene, P35S - *Cauliflower mosaic virus* 35S promoter, *nos* - nopaline synthase gene. *B* - The amount of transcripts analyzed by semi-quantitative RT-PCR with specific primer sets including *BrMDHAR* (the *upper panel*) and *MDHAR* overlapping the *BrMDHAR* and *AtMDHAR1* regions (the *middle panel*). Tubulin was used for normalization. *C* - The *BrMDHAR* expression confirmed by the Western blot analysis using an anti-BrMDHAR antibody. Crude protein extracts (30 μg) were loaded onto a 12 % acrylamide gel. An anti-tubulin antibody was used as house-keeping control. The targeting expressions of *BrMDHAR* to chloroplasts (*D*) and mitochondria (*E*) were confirmed by the Western blot analysis using organellar fractional proteins (20 μg) loaded onto a 15 % acrylamide gel. The *BrMDHAR* expressions were detected by an anti-BrMDHAR antibody in the chloroplastic and mitochondrial fractions. The fractions of chloroplasts and mitochondria were confirmed using cellular component marker protein antibodies, anti-PsbE and anti-COXII, respectively. *F* - The expression of *BrMDHAR* in crude and fractionated proteins was quantified using the *Image J*. WT - wild-type plants, TMR37-3 - transgenic plants overexpressing *BrMDHAR* targeted to chloroplasts and mitochondria, BMR2-2 - transgenic plants overexpressing *BrMDHAR* in cytosol, NORMAL - plants grown under normal conditions for 8 d, FREEZING - plants recovered for 3 d after freezing, TOTAL - crude proteins, CHLO - chloroplast fraction proteins, MITO - mitochondria fraction proteins.

plants, however, the amount of the *BrMDHAR* transcripts in the TG plants with or without targeting signals were increased under the freezing stress when compared to the normal conditions, probably due to the action of the stress-inducible SWPA2 promoter. *BrMDHAR* was expressed in the TG plants also under the normal conditions which was assumed to be caused by ROS produced even under the normal conditions (Noctor and Foyer 1998). The Western blot analysis was performed to ensure that *BrMDHAR* was effectively expressed in chloroplasts and mitochondria through the targeting signal in the TG plants (Fig. 1*C*). Although the *MDHAR* expression was also detected in the WT plants because *A. thaliana MDAHR1* (*AtMDHAR1*) has 92 % identity to *BrMDHAR*, the expression of *BrMDHAR* in the TG plants with or without the targeting signals was 13 and 20 % higher, respectively, than that in the WT plants subjected to freezing. The increased expression of BrMDHAR in the TG plants compared to the WT plants was also observed by semi-quantitative RT-PCR using primer sets amplifying both *AtMDHAR1* and *BrMDHAR* (Fig. 1*B*). The expressions of *BrMDHAR* in the TG plants with or without the targeting signals increased 1.5 and 1.4 times, respectively, under the freezing conditions compared to those in the plants under the normal conditions. To clarify the function of transit peptides, the Western blot analysis was performed using proteins of chloroplasts and mitochondria fractions (Fig. 1*D*). The TG plants overexpressing *BrMDHAR* with the targeting signals showed a much higher expression in chloroplasts (Fig. 1*D*) and mitochondria (Fig. 1*E*). Although the TG plants with the targeting signals showed expression patterns similar to those in the WT plants when total proteins were analyzed, they showed 2.0 and 1.5 times higher expressions, respectively, than in the WT plants when the chloroplast and mitochondrion proteins were analyzed. To verify the organelle fractions, PsbE and COXII antibodies were used (Fig. 1*F*). These results indicate that *BrMDHAR* in the TG plants was dual-targeted to chloroplasts and mitochondria and effectively expressed under the control of the stress-inducible SWPA2 promoter when the TG plants were exposed to the freezing stress.

To confirm that introduced *BrMDHAR* was functional in the TG plants, the MDHAR activity and AsA content were measured. The MDHAR activity (Fig. 2*A*) was 12 and 16 % higher in the TG plants with or without the targeting signals, respectively, than in the WT plants subjected to freezing. Since *Arabidopsis* has 6 isozymes of MDHAR, these increased values for the MDHAR activity in the TG plants represented not only the activity caused by the *BrMDHAR* overexpression, but also the activity of 6 AtMDHAR isozymes. The MDHAR activity was generally higher under the freezing stress when

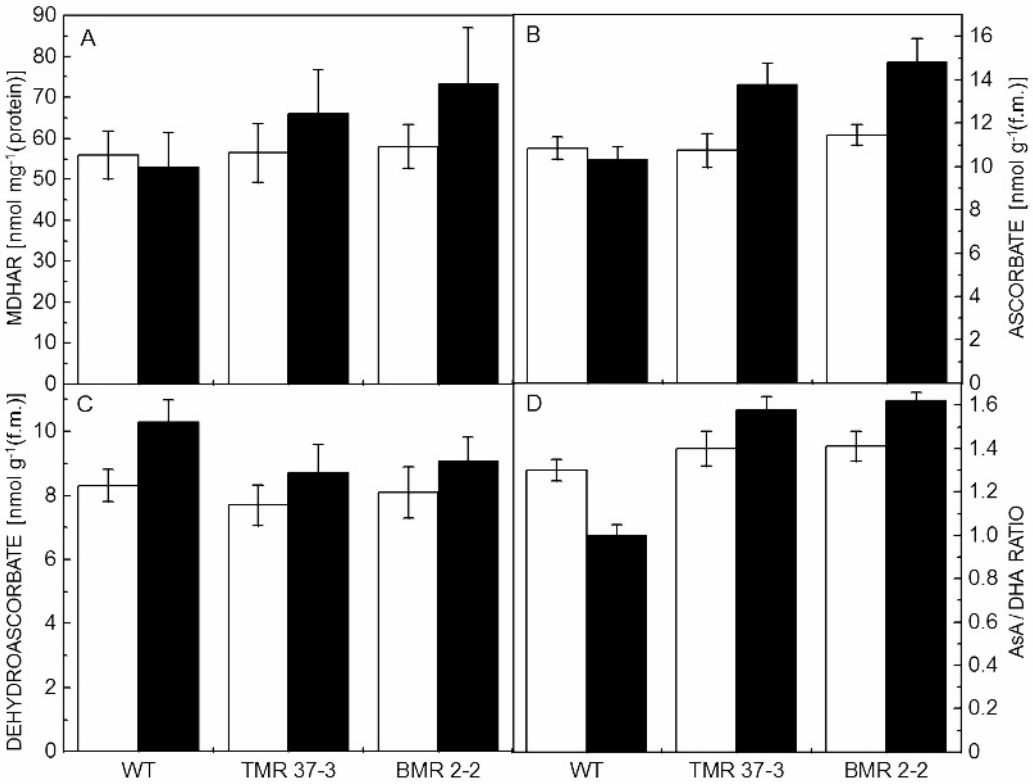

Fig. 2. The MDHAR activity (*A*), the content of ascorbate (*B*) and dehydroascorbate (*C*), and the ratio of AsA to DHA *(D)* in transgenic and wild-type plants. Means ± SD from 3 independent experiments ($P < 0.05$). WT - wild-type plants, TMR37-3 - transgenic plants overexpressing *BrMDHAR* targeted to chloroplasts and mitochondria, BMR2-2 - transgenic plants overexpressing *BrMDHAR* in cytosol; *white bars* - plants grown under normal conditions for 8 d, *black bars* - plants recovered for 3 d after freezing.

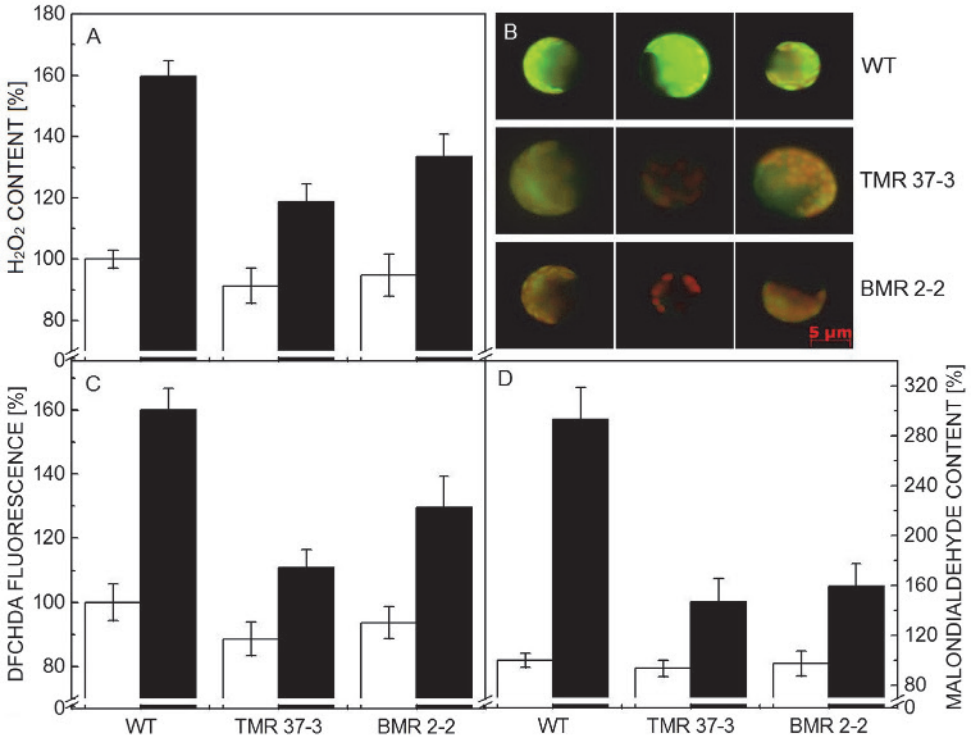

Fig. 3. Redox status and lipid peroxidation in transgenic and wild-type plants. *A* - The content of hydrogen peroxide was determined using the FOX reagent. The oxidation of ferrous ion indicated by xylenol orange was measured spectrophotometrically at 560 nm. *B* - Intracellular ROS was detected in protoplasts extracted from the leaves of transgenic and wild-type plants. Fluorescence induced by hydrogen peroxide in protoplasts was observed by microscopy. *C* - DCFHDA fluorescence was detected using a microplate reader. The increase in fluorescence intensity in response to oxidation was measured in extracts from the transgenic and wild-type plants. *D* - Lipid peroxidation was estimated spectrophotometrically by measuring the malondialdehyde (MDA) content. Means ± SD of three replicates. WT - wild-type plants, TMR37-3 - transgenic plants overexpressing *BrMDHAR* targeted to chloroplasts and mitochondria, BMR2-2 - transgenic plants overexpressing *BrMDHAR* in cytosol; *white bars* - plants grown under normal conditions for 8 d, *black bars* - plants recovered for 3 d after freezing.

compared to the normal conditions, showing a moderate increase of 11.6 and 12.6 % in the TG plants with or without the targeting signals, respectively, whereas a 5.4 % decrease in the WT plants. These results suggest that the ectopic expression of MDHAR was functional in the TG plants with or without the targeting signals. Next, AsA, which is the product of the MDHAR reaction, was measured. The TG plants with the targeting signals showed no significant changes in the AsA content compared to the WT plants under the normal conditions. Under freezing, the AsA content in the WT plants decreased by 5 % compared to that under the normal conditions, but in the TG plants with or without targeting signals increased by 12.8 and 12.9 %, respectively, under the same conditions (Fig. 2*B*). The DHA content increased in response to freezing. Specifically, the WT plants showed a 24 % higher DHA content under freezing than under the normal conditions, whereas the DHA content in the TG plants with or without the targeting signals was 11.3 and 11.2 % higher, respectively, under freezing (Fig. 2*C*). The ratio of AsA to DHA in the TG plants with or without the targeting signals was 58 and 62 % higher, respectively, than in the WT plants subjected to freezing (Fig. 2*D*). These results suggest that AsA recycling by the *BrMDHAR* expression improved AsA homeostasis under the freezing stress.

The content of H_2O_2 was measured by three methods. All the plants had a lower content of H_2O_2 under the normal than under freezing conditions (Fig. 3*A*). The H_2O_2 content of the WT plants was approximately 1.3 times higher than in the TG plants with or without the targeting signals under the freezing stress. The H_2O_2 content in the WT plants increased by 60 % under freezing, but only by 20 and 30 % in the TG plants with or without the targeting signals, respectively. A fluorescence-based method using the H_2O_2-sensitive probe (DCFHDA) confirmed 1.3 and 1.5 times higher H_2O_2 content in the WT than in the TG plants with or without the targeting signals, respectively, under the freezing conditions, whereas the TG plants had only 11 and 8 % lower H_2O_2 content than the WT plants under the normal conditions (Fig. 3*C*). The H_2O_2 content was also analyzed by observing DCFHDA-stained protoplasts under a fluorescence microscope. The cells from the leaves of the TG plants with or without the targeting signals exhibited some green fluorescence in their intracellular spaces. In contrast, WT protoplasts showed much higher fluorescence than protoplasts extracted from

the TG plants (Fig. 3*B*).

MDA as product of lipid peroxidation was measured and under normal conditions, all the plants had a lower MDA content than under the freezing stress. The WT plants had 1.9 and 2.0 times higher MDA content than the TG plants with or without the targeting signals, respectively. The TG plants with or without the targeting signals showed 1.4 and 1.5 times higher MDA content under freezing than under normal conditions, whereas WT plants subjected to freezing stress exhibited 3 times higher MDA content.

These results indicate that the *BrMDHAR* over-expression in the TG plants with or without the targeting signal decreased lipid peroxidation by improving redox homeostasis under the freezing stress.

Chlorophyll content was similar in both the TG and WT plants under the normal conditions. When subjected to the freezing stress, no significant difference was observed in the TG plants with or without targeting signals but the WT plants showed 34 % lower Chl content. The TG plants with or without targeting signals showed approximately 37 % higher Chl content than WT plants

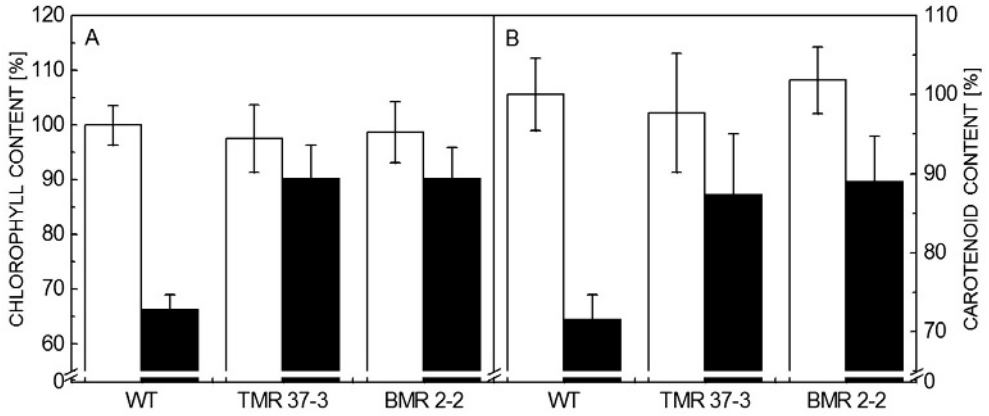

Fig. 4. The chlorophyll and carotenoid content in transgenic and wild-type plants. *Arabidopsis* leaves were harvested and the pigments were extracted from fresh leaf tissue. Means ± SD of 3 replicates. WT - wild-type plants, TMR37-3 - transgenic plants overexpressing *BrMDHAR* targeted to chloroplasts and mitochondria, BMR2-2 - transgenic plants overexpressing *BrMDHAR* in cytosol; *white bars* - plants grown under normal conditions for 8 d; *black bars* - plants recovered for 3 d after freezing.

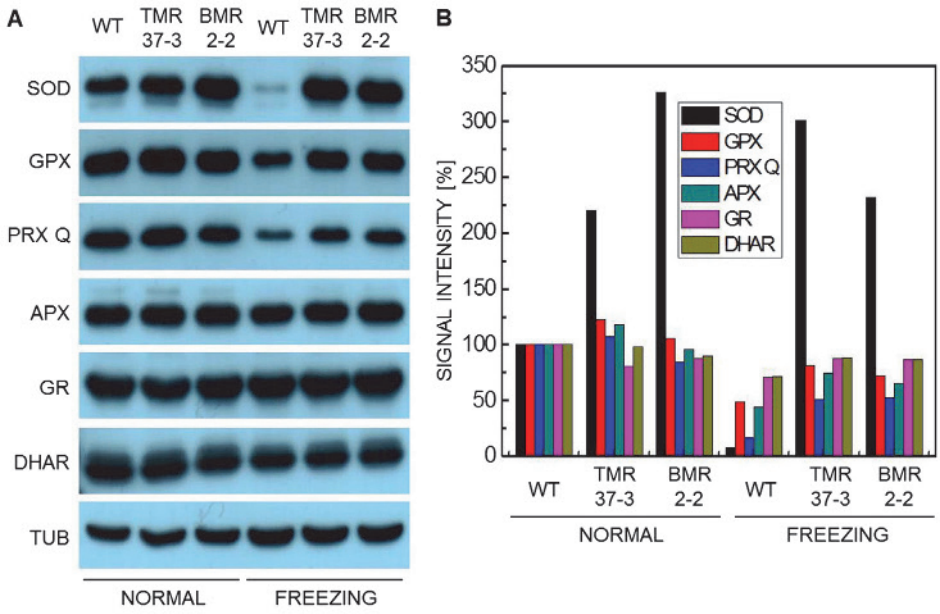

Fig. 5. Changes in the expression of antioxidant enzymes. *A* - The expression of antioxidant enzymes were analyzed by Western blot. Protein quantity was calibrated by a tubulin expression. WT - wild-type plants, TMR37-3 - transgenic plants overexpressing *BrMDHAR* targeted to chloroplasts and mitochondria, BMR2-2 - transgenic plants overexpressing *BrMDHAR* without the signal peptide. *B* - The expressions of antioxidant enzymes were quantified using the *Image J*. SOD - superoxide dismutase, GPX - glutathione peroxidase, PRX Q - peroxiredoxin Q, APX - ascorbate peroxidase, GR - glutathione reductase, DHAR - dehydroascorbate reductase; NORMAL - plants grown under normal conditions for 8 d; FREEZING - plants recovered for 3 d after freezing.

under the freezing stress (Fig. 4*A*). The content of carotenoids was 28 % lower in the WT plants subjected to freezing than under normal conditions, but the TG plants with or without the targeting signals showed no notable changes under freezing conditions even though there was a distinguishable difference of Car content in the TG plants as compared to the WT plants. Although all plants analyzed had similar Car content under normal conditions, the TG plants with or without targeting signals had 22 and 24 % higher Car content than the WT plants under freezing stress (Fig. 4*B*). These results suggest that the *BrMDHAR* overexpression in the TG plants with or without the targeting signals improved photosynthesis ability under the freezing stress due to increased Chl and Car content.

Western blot analysis was performed to examine whether improved redox status could be attributed to the *BrMDHAR* overexpression itself or co-regulation with other antioxidant systems. With the exception of *GR* and *DHAR*, the expression of genes coding examined enzymes increased in the TG plants with or without the targeting signals under freezing more than in the WT plants (Fig. 5*A*). The respective proteins were quantified using *Image J* (Fig. 5*B*). Noticeable induction of antioxidant enzymes in the TG plants did not occur under normal conditions, but the *SOD* was 1.4 and 1.6 times higher in the TG plants with or without the targeting signals, respectively, compared to that in the WT plants. SOD also showed a much higher expression (11.3 and 10.6 times, respectively) in the TG plants with or without the targeting signals subjected to freezing when compared to that in the WT plants. *Prx Q* and *GPX* also showed much higher expressions. Specifically, Prx Q was 1.9 and 2.0 times higher in the TG plants with or without the targeting signals, respectively, than in the WT plants when subjected to freezing, whereas *GPX* was 1.4 and 1.3 times higher in the TG plants with or without the targeting signals, respectively. *APX* showed 1.2 times higher expression in both the TG plants when compared to the WT plants under the freezing stress (Fig. 5). Induction of antioxidant enzymes was more effective in both the TG plants, although the increase in expression differed between the TG plants with and without the targeting signals.

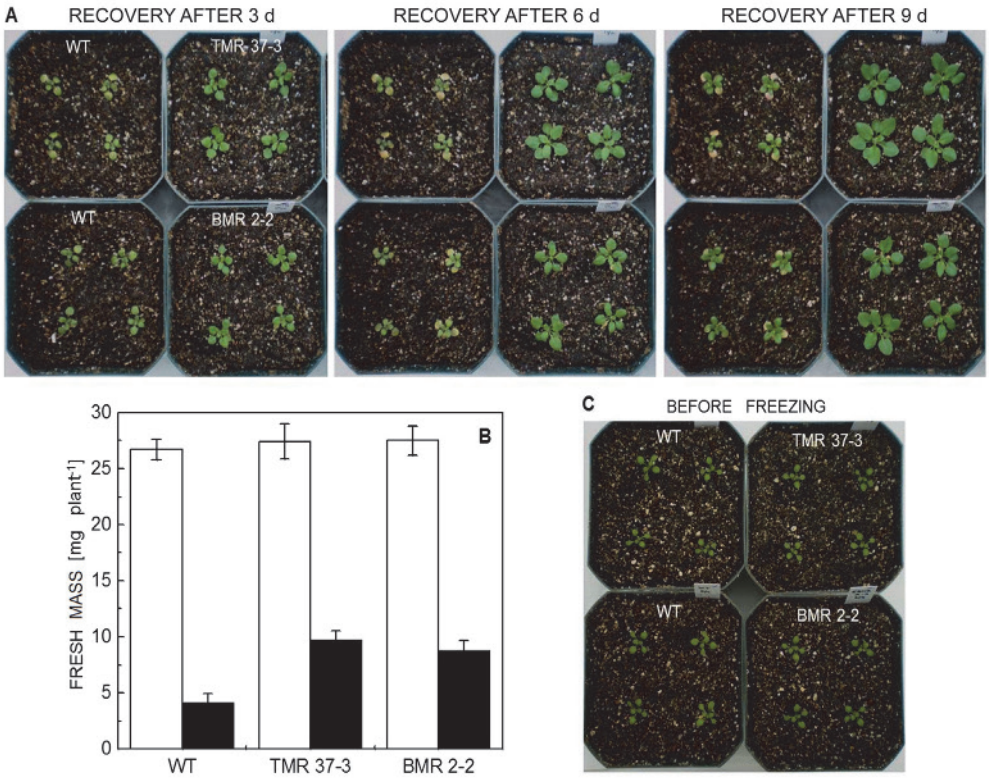

Fig. 6. A freezing stress-tolerance assay in transgenic *Arabidopsis*. *A* - Stress tolerance was assayed by a phenotype analysis of *BrMDHAR*-expressing transgenic plants with and without signal peptides (see Materials and methods for details). *B* - Fresh masses of plants grown for 8 d under normal conditions (*white bars*) and recovered for 3 d after freezing (*black bars*). To confirm freezing tolerance, six independent experiments were conducted. *C* - The phenotype analysis before the freezing treatment. WT - wild-type plants; TMR37-3 - transgenic plants overexpressing *BrMDHAR* targeted to chloroplasts and mitochondria, BMR2-2 - transgenic plants overexpressing *BrMDHAR* in cytosol.

To compare stress adaptation ability, the TG and WT plants were incubated at -5 °C for 16 h, after which they were returned to normal growth conditions (22 °C). After challenging with the freezing stress, the recovery of all plants was observed (Fig. 6*A*). The ability to recover from freezing injury differed between the TG and WT plants as

indicated by differences in phenotype, although there was no noticeable difference between the TG plants with and without the targeting signals. The fresh masses of leaves and shoots greatly decreased in all plants under the freezing stress, however, they were 2.2 and 2.4 times higher in the TG plants with or without the targeting signals, respectively, than in theWT plants under freezing stress (Fig. 6*B*), even though all plants had similar fresh masses under normal conditions (Fig. 6*C*). These results show that the *BrMDHAR* overexpression with or without the targeting signals enhance an acquired tolerance to the freezing stress, indicating that *BrMDHAR* played a crucial role in freezing-induced oxidative stress response.

Discussion

The targeting signal of GR from pea plants is well known for its targeting expression to chloroplasts and mitochondria both *in vivo* and *in vitro* (Creissen *et al.* 1992, 1995, Rudhe *et al.* 2002, Chew *et al.* 2003a). Based on these reports, the transit peptide from pea GR was cloned and its targeting expression in chloroplasts and mitochondria of *Arabidopsis* was confirmed using the transit peptide-fused GFP protein expression system (data not shown). This result shows that a stress response is stimulated if the protein is properly expressed in chloroplasts and mitochondria. To investigate this hypothesis, *BrMDHAR* was fused to the targeting signal of pea GR, and this expression system was introduced into *Arabidopsis* (Fig. 1*A*). The ability of the TG plants to adapt to the stress by the targeting expression of *BrMDHAR* was analyzed by comparing it to the TG plants overexpressing *BrMDHAR* in cytosol which have been shown in our previous experiments to enhance freezing tolerance (unpublished data). The targeted expressions of *BrMDHAR* to chloroplasts and mitochondria were confirmed by semi-quantitative RT-PCR (Fig. 1*B*) at the transcriptional level and by Western blot analysis using fractionated proteins (Fig. 1) and an enzyme assay (Fig. 2*A*) at the translational level. These results indicate that *BrMDHAR* with signal peptides from pea GR was dually targeted to chloroplasts and mitochondria and effectively expressed under the regulation of SWPA2 promoter. Various dual-targeted proteins have also been reported including those targeted to chloroplasts and nucleus (Schwacke *et al.* 2007), chloroplasts and peroxisomes (Reumann *et al.* 2007, Sapir-Mir *et al.* 2008), and chloroplasts and endoplasmic reticulum (Levitan *et al.* 2005) by methods using fluorescent tagging, *in vitro* import, Western blot analysis, and mass spectrometry (Carrie and Whelan 2013). Since the discovery of the first dual-targeted protein in plants in 1995, the number has grown to more than 250 (Carrie and Whelan 2013). Recently, more research has focused on the evolution, conservation, and acquisition of dual-targeting proteins in terrestrial plants (Carrie and Whelan 2013, Xu *et al.* 2013). Much work focused on identifying how and what makes a protein dual-targeted, but there is no information on a stress response under the control of a stress-inducible promoter, such as SWPA2.

Defining the subcellular localization of a protein with accuracy is important, because large-scale phenotype screening, microarray experiments, and protein-protein interactions depend on known information or defined models (Carrie *et al.* 2009b). However, in the case of dual-targeted proteins, there is no systematic analysis. An overexpression of dual-targeting *BrMDHAR* in the TG plants (TMR 37-3) led to an increase of the AsA content through AsA recycling by an increased MDHAR activity (due to the *BrMDHAR* and constitutive *MDHAR* expression) under the freezing stress compared to the WT plants. However, there was no difference in the ratio in the TG plants both with (TMR 37-3) and without (BMR 2-2) signal peptides under the same conditions (Fig. 2). Interestingly, the AsA and DHA content in BMR 2-2 plants was higher than in the TMR 37-3 plants. In addition, the TG plants with the targeted overexpression of *BrMDHAR* (TMR 37-3) showed a better expression of genes coding antioxidant enzymes (*APX*, *SOD*, and *GPX*; Fig. 5) as compared to the TG plants (BMR 2-2) without signal peptides in the presence of the freezing stress, whereas the expressions of other genes analyzed (*GR*, *DHAR*, *Prx Q*) were similar in the plants with and without signal peptides. However, the expressions in both types of the TG plants were higher than in the WT plants. Co-activations of antioxidant enzymes including APX, SOD, and GPX in the TMR 37-3 plants attenuated the cellular H_2O_2 content (Fig. 3) under the freezing stress compared to the BMR 2-2 plants, leading to a reduction of lipid peroxidation (Fig. 3). In particular, redox homeostasis in the TMR 37-3 plants was higher than that in the BMR 2-2 plants. Thus, the ROS content was consistent with AsA homeostasis. The DHA content in the TMR 37-3 plants was lower than in the BMR 2-2 plants. Previous reports suggested that H_2O_2 diffuses immediately from chloroplasts or mitochondria into cytosol. Puntarulo *et al.* (1991) demonstrated that the most important source of cytosolic ROS is the mitochondrial membrane in soybean, and Ishikawa *et al.* (1993) showed the immediate diffusion of ROS from chloroplasts to cytosol. However, we assumed that the chloroplast- and mitochondria-targeted expression of *BrMDHAR* are able to confer enhanced freezing tolerance than the cytosolic expression because diffusion of ROS (such as H_2O_2) from chloroplasts and mitochondria into cytosol before detoxification requires more energy than direct detoxification in chloroplasts or mitochondria. The ROS produced can also operate as second messengers leading to a change in the metabolic process. In addition, improvements in redox status in both types of the TG plants enhanced photosynthetic ability following an increase in the chlorophyll and carotenoid content under

the freezing stress as compared to that in the WT plants (Fig. 4). Our results show that under the regulation of the SWPA2 promoter, *BrMDHAR* dual targeting to chloroplasts and mitochondria scavenged more effectively ROS produced during the freezing stress through the co-activation of other antioxidative systems in the TMR 37-3 plants compared to the BMR 2-2 plants.

Like *BrMDHAR* dual-targeted to mitochondria and chloroplasts, *MDHAR* from *Arabidopsis* (*AtMDHAR6*, acc. No. At1g63940; Chew *et al.* 2003b), *Oryza sativa* (acc. No. Os08g05570; Morgante *et al.* 2009), *Physcomitrella patens* (acc. No. Pp1s90_28V6; Xu *et al.* 2013), and *Picea glauca* (acc. No. BT113791; Xu *et al.* 2013) were dual-targeted to mitochondria and chloroplasts. In terrestrial plants, 107 proteins have been experimentally reported to be dual targeted to mitochondria and chloroplasts, or mitochondria and peroxisomes (Xu *et al.* 2013). These proteins usually play roles in common housekeeping processes which fall into various groups: DNA and RNA repair and binding, protein translation, protein modification, antioxidant activities, *etc*. (Chew *et al.* 2003b, Rovoc-Plavec *et al.* 2008), and now our evidence of *BrMDHAR* involved in the AsA-GSH cycle. Five proteins including methionine (Met) amino peptidase, glutamyl-transfer RNA synthetase, tyrosyl-transfer RNA synthetase, seryl-transfer RNA synthetase, and MDHAR have been shown to be dual targeted in *Arabidopsis*. Mutator S (MutS) homologue 1 was found to be dual-targeted in a number of dicot plants (Xu *et al.* 2013). On the other hand, a number of orthologues to dual-targeted proteins in *Arabidopsis* were also found to be dual-targeted in rice and *Physcomitrella patens* (Carrie *et al.* 2009a, Xu *et al.* 2013). The acquisition of dual-targeting ability may allow organelles to gain additional functions. *Mia40* was found in most eukaryotic species but is well studied in yeast where it is located in the mitochondrial intermembrane space and plays an essential role in the oxidizing and folding proteins (Chacinska *et al.* 2004, Xu *et al.* 2013). It was originally believed that this is the role of Mia40 in all eukaryotic species, however, Mia40 proteins are targeted to peroxisomes in higher plants, such as *Arabidopsis* and *Populus trichocarpa*, but not in lower plants. Mia40 dual-targeted to mitochondria and peroxisomes accompanies superoxide dismutase 1 (Ccs1), copper zinc superoxide dismutase 1 (CSD1), and CSD3 proteins to activate protein folding in *Arabidopsis* (Carrie *et al.* 2010, Xu *et al.* 2013). Indeed, our results show that the *BrMDHAR* expression dual-targeted to chloroplasts and mitochondria in the TG *Arabidopsis* plants implicated freeze-induced oxidative stress defense. In addition, the acquisition of dual-targeted proteins including *BrMDHAR* may represent important means to improve resistance to ROS-induced oxidative stress.

In conclusion, the *BrMDHAR* expression dual-targeted to mitochondria and chloroplasts under the control of SWPA2 promoter conferred tolerance against the freezing stress by improving redox homeostasis and the AsA pool following the co-activation of enzymes involved in the AsA-GSH cycle in TG *Arabidopsis*, leading to an increased total biomass through an enhanced photosynthetic ability. Investigations into the regulation of dual targeting may be the biggest breakthrough but questions, such as whether dual targeting a protein is dependent on the growth stage or development of the plants, or whether stresses affect dual targeting a protein have to be solved.

References

Asada, K.: The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. - Annu. Rev. Plant Physiol. Plant mol. Biol. **50**: 601-639, 1999.

Asada, K., Takahashi M.: Production and scavenging of active oxygen in photosynthesis. - In: Kyle, D.J., Osmond, C.B., Arntzen, C.J. (ed.): Photoinhibition. Pp 227-287. Elsevier Science Publishers, Amsterdam 1987.

Carrie, C., Giraud, E., Duncan, O., Xu, L., Wang, Y., Huang, S., Clifton, R., Murcha, M., Filipovska, A., Rackham, O., Vrielink, A., Whelan, J.: Conserved and novel functions for *Arabidopsis thaliana* MIA40 in assembly of proteins in mitochondria and peroxisomes. - J. biol. Chem. **285**: 36138-36148, 2010.

Carrie, C., Giraud, E., Whelan, J.: Protein transport in organelles: dual tatgeting of proteins to mitochondria and chloroplasts. - FEBS J. **276**: 1187-1195, 2009a.

Carrie, C., Kűhn, K., Murcha, M.W., Duncan, O., Small, I.D., O'Toole, N., Whelan, J.: Approaches to defining dual-targeted proteins in *Arabidopsis*. - Plant J. **57**: 1128-1139, 2009b.

Carrie, C., Small, I.: A re-evaluation of dual-targeting of proteins to mitochondria and chloroplasts. - Biochim. biophys. Acta **1833**: 253-259, 2013.

Carrie, C., Whelan, J.: Widespread dual targeting of proteins in land plants: when, where, how and why. - Plant Signal. Behav. **8** (Suppl.): e25034, 2013.

Chacinska, A., Pfannschmidt, S., Wiedemann, N., Kozjak, V., Sanjuán Szklarz, L.K., Schulze-Specking, A., Truscott, K.N., Guiard, B., Meisinger, C., Pfanner, N.: Essential role of Mia40 in import and assembly of mitochondrial intermembrane space proteins. - EMBO J. **23**: 253-259, 2004.

Chen, H., Nelson, R.S., Sherwood, J.L.: Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. - Biotechniques **16**: 664-668, 1994.

Chew, O., Rudhe, C., Glaser, E., Whelan, J.: Characterization of the targeting signal of dual-targeted pea glutathione reductase. - Plant mol. Biol. **53**: 341-356, 2003a.

Chew, O., Whelan, J., Millar, A.H.: Molecular definition of the ascorbate-glutathione cycle in *Arabidopsis* mitochondria reveals dual-targeting of antioxidant defenses in plants. - J. biol. Chem. **278**: 46869-46877, 2003b.

Clough, S., Bent, A.: Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. - Plant J. **16**: 735-743, 1998.

Creissen, G., Edwards, E.A., Enard, C., Wellburn, A., Mullineaux, P.: Molecular characterization of glutathione reductase cDNAs from pea (*Pisum sativum* L.). - Plant J. **2**: 129-131, 1992.

Creissen, G., Reynolds, H., Xue, Y., Mullineaux, P.: Simultaneous targeting of pea glutathione reductase and a bacterial fusion protein to chloroplasts and mitochondtria in transgenic tobacco. - Plant J. **8**: 167-165, 1995.

Dalton, D., Baird, L.M., Langeberg, L., Tauqher, C.Y., Anyan, W.R., Vance, C.P., Sarath, G.: Subcellular localization of oxygen defense enzymes in soybean (*Glycine max* [L.] Merr.) root nodules. - Plant Physiol. **102**: 481-489, 1993.

Dhindsa, R.S., Plumb-Dhindsa, P., Thorpe, T.A.: Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. - J. exp. Bot. **32**: 93-101, 1981.

Eubel, H., Heazlewood, J.L., Millar, A.H.: Isolation and subfractionation of plant mitochondria for proteomic analysis. - Methods mol. Biol. **355**: 49-62, 2006.

Foreman, J., Demidchik, V., Bothwell, J.H., Mylona, P., Miedema, H., Torres, M.A., Linstead, P., Costa, S., Brownlee, C., Jones, J.D., Davies, J.M., Dolan. L.: Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. - Nature **422**: 442-446, 2003.

Foyer, C.H., Halliwell, B.: The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. - Planta **133**: 21-25, 1976.

Fridovich, I.: Biological effects of the superoxide radical. - Arch. Biochem. Biophys. **247**: 1-11, 1986.

Gillespie, K., Ainsworth, E.: Measurement of reduced, oxidized and total ascorbate content in plants. - Nat. Protocols **2**: 871-874, 2007.

Halliwell, B.: Oxidative stress and neurodegeneration: where are we now? - J. Neurochem. **97**: 1634-1658, 2006.

Halliwell, B., Gutteridge, J.M.: Role of free radicals and catalytic metal ions in human disease: an overview. - Methods Enzymol. **186**: 1-85, 1990.

Harlow, E., Lane, D.: Antibodies: A Laboratory Manual. - Cold Spring Harbor Press, New York 1988.

Heath, R., Packer, L.: Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. - Arch. Biochem. Biophys. **125**: 189-198, 1968.

Hossain, M., Nakano, Y., Asada, K.: Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. - Plant Cell Physiol. **25**: 385-395, 1984.

Ishikawa, T., Takeda, T., Shigeoka, S., Hirayama, O., Mitsunaga, T.: Hydrogen peroxide generation in organelles of *Euglena gracilis*. - Phytochemistry **33**: 1297-1299, 1993.

Jansson, S.: The light-harvesting chlorophyll *a*/*b*-binding proteins. - Biochim. biophys. Acta **1184**: 1-19, 1994.

Jezek, P., Hlavatá, L.: Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. - Int. J. Biochem. Cell Biol. **37**: 2478-2503, 2005.

Jimenez, A., Hernandez, J.A., Del Rio, L.A., Sevilla, F.: Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves. - Plant Physiol. **114**: 275-284, 1997.

Karimi, M., Inze, D., Depicker, A.: GATEWAY vectors for *Agrobacterium*-mediated plant transformation. - Trends Plant Sci. **7**: 193-195, 2002.

Kim, K.Y., Kwon, S.Y., Lee, H.S., Hur, Y., Bang, J.W., Kwak, S.S.: A novel oxidative stress-inducible peroxidase promoter from sweetpotato: molecular cloning and characterization in transgenic tobacco plants and cultured cells. - Plant mol. Biol. **51**: 831-838, 2003.

Levitan, A., Trebitsh, T., Kiss, V., Pereq, Y., Danqoor, I., Danon, A.: Dual targeting of the protein disulfide isomerase RB60 to the chloroplast and the endoplasmic reticulum. - Proc. nat. Acad. Sci. USA **102**: 6225-6230, 2005.

Lichtenthaler, H.: Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. - Methods Enzymol. **148**: 350-382, 1987.

Mittler, R.: Oxidative stress, antioxidants and stress tolerance. - Trends Plant Sci. **7**: 405-410, 2002.

Mittova, V., Tal, M., Volokita, M., Guy, M.: Up-regulation of the leaf mitochondrial and peroxisomal antioxidative systems in response to salt-induced oxidative stress in the wild salt-tolerant tomato species *Lycopersicon pennellii*. - Plant Cell Environ. **26**: 845-856, 2003.

Mizuno, M., Kamei, M., Tsuchida, H.: Ascorbate peroxidase and catalase cooperate for protection against hydrogen peroxide generated in potato tubers during low-temperature storage. - Biochem. mol. Biol. Int. **44**: 717-726, 1998.

Møller, I.M.: Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. - Annu. Rev. Plant Physiol. Plant mol. Biol. **52**: 561-591, 2001.

Moon, H., Lee, B., Choi, G., Shin, D., Prasad, D.T., Lee, O., Kwak, S.S., Kim, D.H., Nam, J., Bahk, J., Hong, J.C., Lee, S.Y., Cho, M.J., Lim, C.O., Yun, D.J.: NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. - Proc. nat. Acad. Sci. USA **100**: 358-363, 2003.

Morgante, C.V., Rodrigues, R.A., Marbach, P.A., Borgonovi, C.M., Moura, D.S., Silva-Filho, M.C.: Conservation of dual-targeted proteins in *Arabidopsis* and rice points to a similar pattern of gene-family evolution. - Mol. Genet. Genomics **281**: 525-538, 2009.

Navrot, N., Rouhier, N., Gelhaye, E., Jacquot, J.: Reactive oxygen species generation and antioxidant systems in plant mitochondria. - Physiol. Plant. **129**: 185-195, 2007.

Njus, D., Kelley, P.M.: Vitamins C and E donate single hydrogen atoms *in vivo*. - FEBS Lett. **284**: 147-151, 1991.

Noctor, G., Foyer, C.: Ascorbate and glutathione: keeping active oxygen under control. - Annu. Rev. Plant Physiol. Plant mol.Biol. **49**: 249-279, 1998.

Obara, K., Sumi, K., Fukuda, H.: The use of multiple transcription starts causes the dual targeting of *Arabidopsis* putative monodehydroascorbate reductase to both mitochondria and chloroplasts. - Plant Cell physiol. **43**: 697-705, 2002.

Prasad, T.K., Anderson, M.D., Martin, B.A., Stewart, C.R..: Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. - Plant Cell **6**: 65-74, 1994.

Puntarulo, S., Galleano, M., Sanchez, R.A., Boveris, A.: Superoxide anion and hydrogen peroxide metabolism in soybean embryonic axes during germination. - Biochim. biophys. Acta **1074**: 277-283, 1991.

Reumann, S., Babujee, L., Ma, C., Wienkoop, S., Siemsen, T., Antonicelli, G.E., Rasche, N., Lűder, F., Weckwerth, W., Jahn, O.: Proteome analysis of *Arabidopsis* leaf peroxisomes reveals novel targeting peptides, metabolic pathways, and defense mechanisms. - Plant Cell **19**: 3170-3193, 2007.

Rokov-Plavec, J., Dulic, M., Duchêne, A.M., Weygand-Durasevic, I.: Dual targeting of organellar seryl-tRNA

synthetase to maize mitochondria and chloroplasts. - Plant Cell Rep. **27**: 1157-1168, 2008.

Rudhe, C., Clifton, R., Whelan, J., Glaser, E.: N-terminal domain of the dual-targeted pea glutathione reductase signal peptide controls organellar targeting efficiency. - J. mol. Biol. **324**: 577-585, 2002.

Sapir-Mir, M., Mett, A., Belausov, E., Tal-Meshulam, S., Frydman, A., Gidoni, D., Eyal, Y.: Peroxisomal localization of *Arabidopsis* isopentenyl diphosphate isomerases suggests that part of the plant isoprenoid mevalonic acid pathway is compartmentalized to peroxisomes. - Plant Physiol. **148**: 1219-1228, 2008.

Schroeder, J.I., Allen, G.J., Hugouvieux, V., Kwak, J.M., Waner, D.: Guard cell signal transduction. - Annu. Rev. Plant Physiol. Plant mol. Biol. **52**: 627-658, 2001a.

Schroeder, J.I., Kwak, J.M., Allen, G.J.: Guard cell abscisic acid signalling and engineering drought hardiness in plants. - Nature **410**: 327-330, 2001b.

Schwacke, R., Fischer, K., Ketelsen, B., Krupinska, K., Krause, K.: Comparative survey of plastid and mitochondrial targeting properties of transcription factors in *Arabidopsis* and rice. - Mol. Genet. Genomics **277**: 631-646, 2007.

Scrutton, N.S., Berry, A., Perham, R.N.: Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. - Nature **343**: 38-43, 1990.

Shultz, R., Settlage, S.B., Hanley-Bowdoin, L., Thompson, W.F.: A trichloroacetic acid-acetone method greatly reduces infrared autofluorescence of protein extracts from plant tissue. - Plant mol. Biol. Rep. **23**: 405-409, 2005.

Spickett, C.M., Smirnoff, N., Pitt, A.R.: The biosynthesis of erythroascorbate in *Saccharomyces cerevisiae* and its role as an antioxidant. - Free Radicals Biol. Med. **28**: 183-192, 2000.

Sweetlove, L., Heazlewood, J.L., Herald, V., Holtzapffel, R., Day, D.A., Leaver, C.J., Milliar, A.H.: The impact of oxidative stress on *Arabidopsis* mitochondria. - Plant J. **32**: 891-904, 2002.

Wagner, A.M.: A role for active oxygen species as second messengers in the induction of alternative oxidase gene expression in *Petunia hybrida* cells. - FEBS Lett. **368**: 339-342, 1995.

Wang, H., Joseph, J.: Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. - Free Radicals Biol. Med. **27**: 612-616, 1999.

Wolff, S.: Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. - Methods Enzymol. **233**: 182-189, 1994.

Xu, L., Carrie, C., Law, S.R., Murcha, M.W., Whelan, J.: Acquisition, conservation, and loss of dual-targeted proteins in land plants. - Plant Physiol. **161**: 644-662, 2013.

Yoo, S.D., Cho, Y.H., Sheen, J.: *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. - Nat. Protoc. **2**: 1565-1572, 2007.