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Increased tolerance to photooxidative stress by overexpression of mitochondrial superoxide dismutase in transgenic tobacco

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Under abiotic stress conditions, plants suffer from oxidative damage caused by increased generation of reactive oxygen species (ROS). Although chloroplasts are the main source of ROS generation in plant cells, changes in ROS level in mitochondria affect plant stress response. In this study, we investigated whether overexpression of mitochondrial manganese superoxide dismutase (Mn-SOD) can enhance tolerance to photooxidative stress. We produced transgenic tobacco plants overexpressing rice Mn-SOD under the control of cauliflower mosaic virus 35S promoter, and assessed the stress tolerance of those plants. The transgenic lines showed higher total SOD activity by 2.8 to 5.2 fold than wild type plants. Chlorophyll fluorescence measurement revealed that the stress-induced inhibition of photosystem II was alleviated under high light, chilling and heat stresses in the transgenic lines. This result indicates that the Mn-SOD contributes to the defense against photooxidative damages in chloroplasts under these stress conditions.

Keywords: Photooxidative stress, reactive oxygen, superoxide dismutase, stress tolerance, tobacco

Introduction

Due to their sessile nature, plants are constantly exposed to various abiotic stresses. In higher plants, the photosynthetic electron transport system in chloroplasts produces reactive oxygen species (ROS). The production of ROS in chloroplasts is enhanced under abiotic stress conditions such as high light, drought, and chilling, and plants suffer from oxidative damages under prolonged stress conditions[1-2].

In order to cope with oxidative injuries, plants are equipped with a number of ROS scavenging enzymes. Among those enzymes, superoxide dismutase (SOD: EC 1.15.1.1) plays a central role in ROS scavenging by catalyzing the decomposition of superoxide radical to hydrogen peroxide and molecular oxygen[2-3]. SOD isoforms are classified by their metal cofactors: copper/zinc (Cu/Zn), manganese (Mn) and iron (Fe) forms and they are distributed in different subcellular compartments[3-4]. The importance of SODs in stress defense has been demonstrated by overexpression of SODs in transgenic plants, which resulted in the enhanced tolerance to oxidative and abiotic stresses[3-4].

Although chloroplasts are the main source of ROS generation within plant cells and ROS production in mitochondria is low compared with chloroplasts[5], ROS homeostasis in mitochondria has large influences on cellular signaling and stress response in plants[6]. Previous studies demonstrated that alteration in electron transport and ROS production in mitochondria affects the expression of genes involved in stress defense and antioxidant enzyme activities[7-10]. The importance of mitochondrial Mn-SOD in ROS scavenging has been suggested since Mn-SOD is not inhibited by hydrogen peroxide, which is a product of the SOD reaction, whereas Cu/Zn- and Fe-SODs are sensitive to hydrogen peroxide. Physiological significance of Mn-SOD in stress defense is demonstrated by overexpression of mitochondrial Mn-SOD, which leads to elevation of stress tolerance against abiotic stresses (salt, cold, heat, drought, aluminum and cadmium) and growth enhancement[11-20]. Overexpression of mitochondrial Mn-SOD can also elevate tolerance to oxidative stress induced by methyl viologen or H_2O_2[13,15,20-21]. However it is

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unclear whether Mn-SOD overexpressing plants have increased tolerance under naturally occurring photooxidative stress since stress tolerance of those plants has not been tested under high light condition. Therefore we set out to examine whether mitochondrial Mn-SOD contributes to the defense against photooxidative stress. For this purpose, in this study we produced transgenic tobacco plants overexpressing mitochondrial Mn-SOD from rice (rice *sodA*) and examined their photooxidative stress tolerance.

Materials and Methods

Production of Transgenic Tobacco Plants

For over expression of rice *sodA1* (RAP-DB gene ID: Os05g0323900) in transgenic tobacco, the entire coding region of *sodA1* was excised from the cDNA plasmid (Accession No. L19436)[22] by *Eco*RI digestion and cloned into the pGEMEX-1 vector (Promega, Madison, WI, USA). Then the *Bam*HI - *Sac*I fragment was excised and integrated into the *Bam*HI and *Sac*I sites of pBI121 in the place of the β-glucuronidase coding sequence. Thus the *sodA1* coding region was fused to the downstream of the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 1A). The resulting T-DNA plasmid was used for *Agrobacterium*-mediated transformation of tobacco (*Nicotiana tabacum* L. cv Petit Havana SR1) as described previously[23]. The regenerated plants (M14 lines) were grown on soil in an incubator under a 16 h light/8 h dark cycle at 15 μmol photons m^{-2} s^{-1} at 25°C to yield seeds.

Seedlings of T_1 generation of M14 lines and the wild type (WT) were grown aseptically on Murashige & Skoog (MS) agar plates at 25°C under a 16 h light/8 h dark cycle at 15 μmol photons m^{-2} s^{-1} for 2 to 4 weeks. The transgenic lines were selected on plates containing 100 mg/L kanamycin. After 2 days of acclimation to the ambient environment, the seedlings were transplanted on soil and grown under the same condition as described above.

Western Blot Analysis and SOD Assay

Protein extracts were prepared from green leaves of wild type (WT) and the transgenic lines at 9 weeks after sowing. Leaf samples were homogenized on ice with an extraction buffer containing 50 mM potassium phosphate (pH 7.6), 0.1 mM EDTA, 0.1% (v/v) Triton X-100. The homogenate was centrifuged at 20,000 x g for 15 min at 4°C and the supernatant was recovered. The protein content was determined by the Bradford method. Twenty μg of proteins was fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immun-Blot PVDF Membrane; Bio-Rad, Hercules, CA, USA). Mn-SOD protein was detected using the rabbit polyclonal antibody against rat Mn-SOD (Enzo Life Sciences, Farmingdale, NY, USA) as a primary antibody at a 1: 5,000 dilution. Alkaline phosphatase-conjugated anti-rabbit IgG antibody (Promega) was used as a secondary antibody at a 1: 50,000 dilution. Signal bands were stained with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT color development substrate; Promega). Total SOD activity was measured as described previously[24].

Analysis of Stress Tolerance of the Transgenic Tobacco Plants

To assess the tolerance to chilling and heat stresses, 6 to 9 week old WT and the M14 transgenic plants

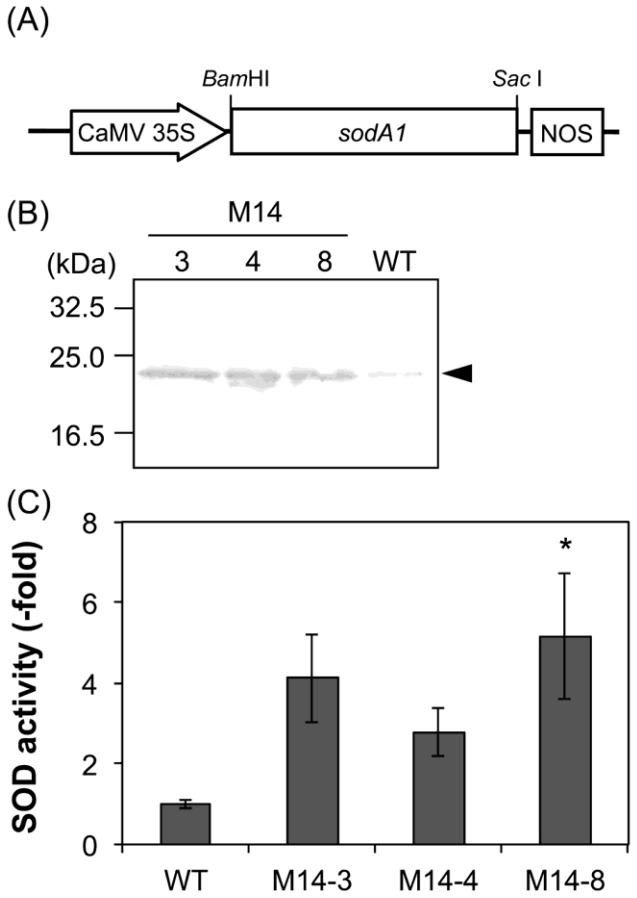

Fig. 1 — Overexpression of Mn-SOD in transgenic tobacco plants transformed with the rice Mn-SOD gene. (A), The transgene construct for overexpression of Mn-SOD. CaMV, CaMV 35S promoter; *sodA1*, rice Mn-SOD cDNA; NOS, terminator of nopalin synthase (NOS) gene. (B), Western blot analysis of Mn-SOD protein in T_1 plants of the transgenic tobacco (M14). WT, wild type. (C), Total SOD activity in T_1 plants of the transgenic tobacco. Data are presented as values relative to WT (n=6 ± SE). Asterisk indicates statistical significance between WT and the transgenic line (Dunnett's test: $p < 0.05$).

were incubated in a growth chamber controlled at 150 μmol photons m^{-2} s^{-1} at 10 or 45°C. For high light treatment, leaf disks prepared from WT and the transgenic lines were illuminated at 700 μmol photons m^{-2} s^{-1} with a halogen lamp on a moist filter paper. All the stress treatments described above were carried out under constant illumination. The leaves were sampled and the chlorophyll fluorescence of photosystem II (PS II) was measured by a pulse-amplitude modulation (PAM) fluorometer (PAM-101, Waltz, Effeltrich, Germany). After the dark adaptation for 30 min, the minimum fluorescence yield (*F*o) was determined. Samples were illuminated subsequently with a saturating pulse of white light at 2,600 μmol photon m^{-2} s^{-1} for 0.8 s to determine the maximal fluorescence yield (*F*m). The maximal quantum yield of PS II (*F*v/*F*m) was determined from the following equation: *F*v/*F*m = (*F*m –*F*o)/ *F*m.

Measurement of Hydrogen Peroxide Content

WT and the M14 transgenic plants at 7 - 8 weeks after sowing were treated with chilling stress at 10°C for 20 h as described above. Green leaves were sampled and frozen immediately in liquid nitrogen. Frozen samples were ground and homogenized in 6% (w/v) perchloric acid with active charcoal powders. The homogenate was centrifuged at 20,000 x g for 10 min at 4°C and the supernatant was recovered. To measure hydrogen peroxide content, the extract was neutralized with 0.5 M NH_4OH and used for a hydrogen peroxide assay by homovanillic acid oxidation[25].

Results and Discussion

We introduced a transgene construct expressing the rice Mn-SOD gene under the control of the CaMV 35S promoter into tobacco (Fig. 1A). We obtained 10 regenerated primary transformants and found that 4 plants showed higher total SOD activity than WT (data not shown). We selected 3 lines for propagation and further analysis. In order to confirm the overexpression of Mn-SOD in the T_1 plants of the transgenic lines, we performed western blot analysis using an antibody against Mn-SOD (Fig. 1B). The result showed that all the transgenic lines contained higher level of Mn-SOD protein than WT, indicating the overexpression of Mn-SOD in the transformants. Also the transgenic lines showed higher total SOD activity by 2.8 to 5.2 fold than WT (Fig. 1C).

Next, we examined photooxidative stress tolerance of the transgenic lines under abiotic stress conditions. In order to assess the photooxidative damages of photosynthetic machinery in chloroplasts, we monitored chlorophyll fluorescence of PS II under high light (Fig. 2A), chilling (Fig. 2B) and heat (Fig. 2C) stresses. These stresses decreased the *F*v/*F*m ratios of both WT and the transgenic lines, indicating that the stress treatments inhibited the photochemical reaction of PS II. However, the decrease in *F*v/*F*m was significantly alleviated in the

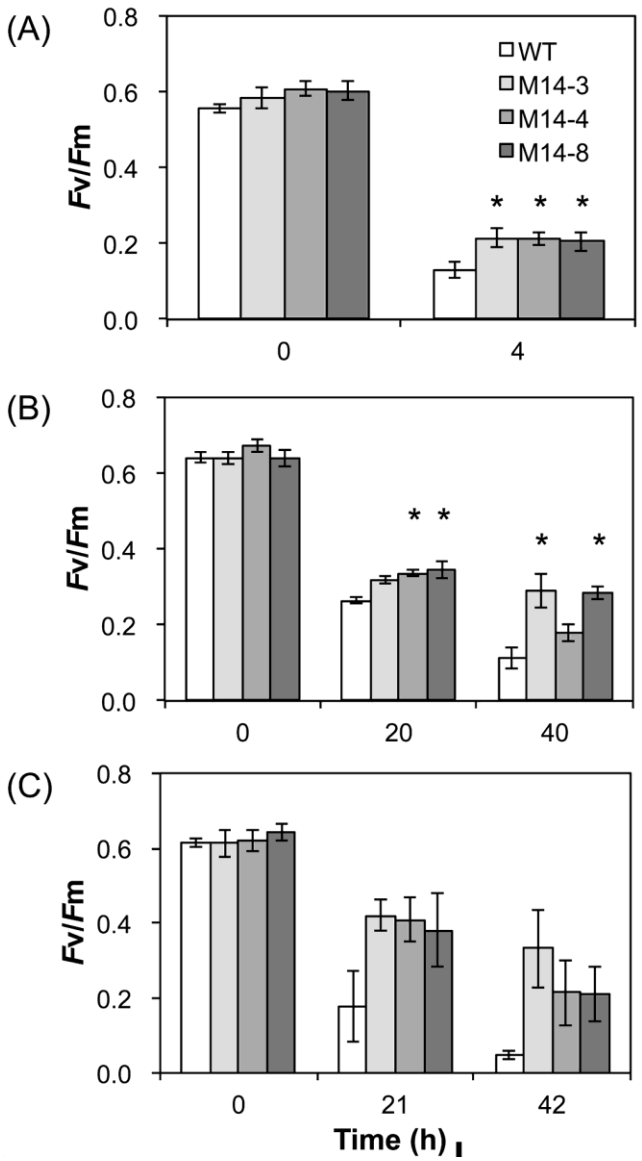

Fig. 2 — Enhanced stress tolerance of Mn-SOD overexpressing tobacco. The transgenic lines and WT were treated with (A) high light (700 μmol photons m^{-2} s^{-1}), (B) chilling (10°C) and (C) heat (45°C) for the indicated period. Chlorophyll fluorescence of PS II was measured, and the decline of *F*v/*F*m was monitored. Data shown are means ± SE (n = 4-16). Asterisks indicate statistical significance between WT and the transgenic lines (Dunnett's test: $p < 0.05$).

Mn-SOD expressing lines compared with WT under high light stress (Fig. 2A). Also the *F*v/*F*m ratio was higher in the transgenic lines than WT under chilling and heat stresses (Fig. 2B & 2C). These results demonstrate that the overexpression of mitochondrial Mn-SOD resulted in the increased tolerance to photooxidative stress in chloroplasts. In contrast to our current results, no elevation of photooxidative stress tolerance was observed under high light condition in transgenic plants overexpressing chloroplastic Fe-SOD in a previous study[26]. Although Mn-SOD is localized in mitochondria, our results suggest that Mn-SOD has a more important role in stress defense than Fe-SOD. In addition, the results also imply that Mn-SOD contributes to the defense against photooxidative damages in chloroplasts.

Next, we tried to gain insight into the mechanisms underlying the protective effects of Mn-SOD overexpression. We assumed a possible influence of Mn-SOD on the stress tolerance. Hydrogen peroxide functions as a signaling molecule that regulates cellular stress response[27-28]. Since hydrogen peroxide is a product of the SOD reaction, it is hypothesized that overexpression of Mn-SOD could elevate the cellular hydrogen peroxide level, inducing the up-regulation of the defense response against abiotic stresses. In order to examine this possibility, we measured hydrogen peroxide content in Mn-SOD overexpressing lines under control and chilling stress conditions. We observed significant elevation of hydrogen peroxide level by chilling treatment in the transgenic lines (Fig. 3; $p<0.05$, *t*- test for the control samples vs chilling samples of each transgenic line). However, there were no significant differences between WT and the transgenic plants under both non-stressed and stressed conditions. Previous studies revealed that changes in mitochondrial ROS production affect gene expression involved in stress responses, as well as abiotic and biotic stress tolerance[8-9]. Although we could not detect any alteration in the cellular hydrogen peroxide level in our experiment, it is likely that local changes in ROS homeostasis might occur in mitochondria by the overexpression of Mn-SOD in the transgenic lines, which in turn would change global gene expression to enhance abiotic stress tolerance.

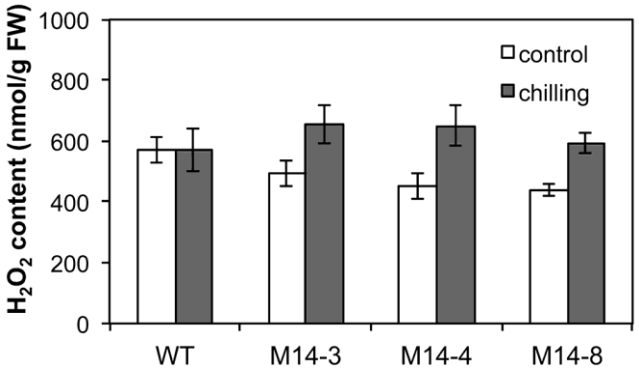

Fig. 3 — Hydrogen peroxide content in the Mn-SOD−overexpressing tobacco treated with chilling stress (10°C). Open and filled bars indicate control and treated samples, respectively. Data shown are means ± SE (n = 4-6). No significant difference was observed between WT and the transgenic lines by Dunnett's test.

In conclusion, our results revealed that the overexpression of mitochondrial Mn-SOD in transgenic tobacco alleviated the inhibition of PS II under high light, chilling and heat stresses. These findings indicate that Mn-SOD contributes to the protection of chloroplasts from photooxidative damages under these abiotic stresses.

Acknowledgments

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