Journal of Plant Physiology **162** (2005) 465—472

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JOURNAL OF PLANT PHYSIOLOGY

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Enhanced drought tolerance of transgenic rice plants expressing a pea manganese superoxide dismutase

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Received 27 April 2004; accepted 23 September 2004

KEYWORDS
Drought;
Oryza sativa;
Oxidative stress;
Superoxide dismutase;
Transgene

Summary

We investigated the role that manganese superoxide dismutase (MnSOD), an important antioxidant enzyme, may play in the drought tolerance of rice. MnSOD from pea (*Pisum sativum*) under the control of an oxidative stress-inducible *SWPA2* promoter was introduced into chloroplasts of rice (*Oryza sativa*) by *Agrobacterium*-mediated transformation to develop drought-tolerant rice plants. Functional expression of the pea *MnSOD* in transgenic rice plants (T_1) was revealed under drought stress induced by polyethylene glycol (PEG) 6000. After PEG treatment the transgenic leaf slices showed reduced electrolyte leakage compared to wild type (WT) leaf slices, whether they were exposed to methyl viologen (MV) or not, suggesting that transgenic plants were more resistant to MV- or PEG-induced oxidative stress. Transgenic plants also exhibited less injury, measured by net photosynthetic rate, when treated with PEG. Our data suggest that SOD is a critical component of the ROS scavenging system in plant chloroplasts and that the expression of MnSOD can improve drought tolerance in rice.

Abbreviations: APX, ascorbate peroxidase; GUS, β-glucuronidase; MV, methyl viologen; NBT, nitro blue tetrazolium chloride; PEG, polyethylene glycol; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction; SOD, superoxide dismutase; UBI, ubiquitin

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doi:10.1016/j.jplph.2004.09.009

Introduction

Exposure of plants to certain environmental stresses can lead to the generation of reactive oxygen species (ROS), including superoxide anion radicals (O_2^-), hydroxyl radicals (•OH), hydrogen peroxide (H_2O_2) and singlet oxygen (O_2^1). Injury caused by ROS, known as oxidative stress, is one of the major damaging factors in plants exposed to environmental stresses such as drought (Price et al., 1989), desiccation (Senaratna et al., 1985a, b), extreme temperatures (Kendall and McKersie 1989; McKersie et al., 1993), high light intensity (Fryer et al., 2002), salinity (Hernandez et al., 1995), ozone (Van Camp et al., 1994), anoxia (Monk et al., 1989), the herbicide paraquat (Bowler et al., 1991) and pathogens (Mehdy, 1994). Chloroplasts are particularly susceptible to ROS because of the relatively high concentration of oxygen that reacts with electrons that escape from the photosynthetic electron transfer system (Foyer et al., 1994). ROS can affect a variety of biological macromolecules, lead to severe cellular damage, inhibit photosynthesis and hence reduce the yield of crops.

Plants have evolved a wide range of enzymatic and non-enzymatic mechanisms to scavenge ROS. Among enzymatic mechanisms superoxide dismutase (SOD) plays an important role. SOD (EC 1.15.1.1) catalyzes the dismutation of two molecules of superoxide into oxygen and hydrogen peroxide (Fridovich, 1975), the first step in ROS scavenging systems. SODs are classified according to their metal cofactor as FeSOD, MnSOD and Cu/ZnSOD. Plants generally contain Cu/ZnSOD in the cytosol, FeSOD and/or Cu/ZnSOD in chloroplasts, and MnSOD in mitochondria (Bowler et al., 1994).

The level of enhancement of SOD activity under stress conditions resulting from introducing SOD genes into plants may result in more efficient elimination of ROS. Several transgenic plants with extragenetic SODs resulting in over-expression primarily in the chloroplasts have been reported. SOD transformants varied in their protective response to oxidative stresses, induced by MV or environmental stresses such as chilling, ozone, water deficit and salt (Foyer et al., 1994; Allen, 1995). In some transgenic plants significant improvements were achieved (Bowler et al., 1991; McKersie et al., 1993; Perl et al., 1993; Sen Gupta et al., 1993a, b; Van Camp et al., 1994, 1996; McKersie et al., 1999), whereas in others no improvements in stress tolerance were observed (Tepperman and Dunsmuir, 1990; Pitcher et al., 1991; Payon et al., 1997). This may be attributed to the complexity of the ROS detoxification system (Kwon et al., 2002) and the differences of SOD isoenzymes. Constitutive over-expression of DREB1A led to increased expression of rd29A, rd17, cor6.6, cor15a, erd10, and kin1, resulting in a marked increase in the transgenic plant tolerance to freezing, water stress, and salinity. In the absence of stress, however, plant growth was severely retarded, particularly in the strongest-expressing lines. In contrast, the rd29A–DREB1A construct, which was expressed only in response to cold and water stress, conferred similar levels of stress tolerance, but with little growth retardation (Kasuga et al., 1999).

Rice (*Oryza sativa*) is the major food crop in Asia, but stress conditions such as drought often cause severe yield loss. The observation that plants under drought stress generate reactive oxygen species (Price et al., 1989) and the fact that transgenic alfalfa expressing MnSOD has reduced injury from water deficit (McKersie et al., 1996) prompted us to study whether rice expressing foreign SOD under the control of a stress inducible promoter would improve drought tolerance. In this study, we developed transgenic rice plants that express pea *MnSOD* in chloroplasts under the control of a stress-inducible *SWPA*2 promoter (Kim et al., 2003), and compared tolerance to oxidative stress and photosynthesis rates between wild type and T_1 transgenic plants under drought stress mediated by PEG.

Materials and methods

Plasmid construction and rice transformation

A chimeric chloroplast targetted MnSOD gene construct in pRTL2 (Schake, 1995), which consisted of enhanced CaMV 35S promoter, tobacco etch virus (TEV) 5′-untranslated sequence, transit peptide (TP) for chloroplast targeting from pea *Cu/ZnSOD*, pea *MnSOD* cDNA and CaMV 35S terminator, was used for construction of *pSWPA*2pro::*MnSOD*. In the *pSWPA*2pro::*MnSOD*, the enhanced CaMV 35S promoter was replaced with an oxidative stress-inducible promoter, -1314 *SWPA*2 promoter (Kim et al., 2003). The transgene cassette was transformed into pCAMBIA1301 and the resulting recombinant plasmid pCAMBIA1301-*pMnSOD* was used for plant transformation (Fig. 1).

Transformation of rice (*Oryza sativa* L. cv. Zhonghua 11) was achieved by co-cultivation of calli derived from immature embryo scutella with *Agrobacterium tumefaciens* EHA105 containing pCAMBIA1301-*pMnSOD* (Hiei et al., 1994). The transgenic plants regenerated from hygromycin

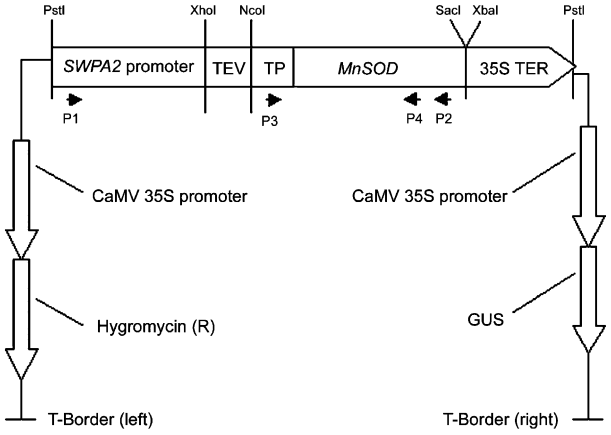

Figure 1. Schematic representation of pCAMBIA1301-pMnSOD construct. The pea MnSOD was fused to an oxidative stress-inducible *SWPA2* promoter, a translation leader from tobacco etch virus (TEV), and a transit peptide for chloroplast targeting (TP). Transgenic plants were selected with hygromycin and GUS expression. P1/P2 and P3/P4 are two pairs of primers used for PCR and RT-PCR, respectively.

resistant calli by somatic embryogenesis (Horsch et al., 1985).

Transgenic plants screening

The putative T_0 transgenic plants were screened for the presence of T-DNA by GUS staining of leaves, and confirmed by polymerase chain reaction (PCR) analysis using primers P1 (5′-TTG CCA AAT CCT ATC ATA CAC-3′) and P2 (5′-GTC GTC CCA AGT TCC ACA-3′). P1 and P2 are matched to the *SWPA2* promoter and to *pMnSOD*, respectively (Fig. 1). PCR products were analyzed on 1% agarose gel containing ethidium bromide. In total, 15 lines of PCR positive T_0 plants were obtained and the progenies from two of them (T_1) were selected for further research. Every T_1 plant used in this work was checked again by GUS staining before physiological assays to ensure the presence of the transgene.

Growth condition and stress treatment

WT and T_1 transgenic rice plants were cultivated in the same container filled with Kimula B nutrient solution (Ni, 1985) in the greenhouse at approximately 28/24 °C (day/night) and in 14 h (from 6:00 to 20:00) light intensity of 200 μmol/m^2/s provided by xenon lamp.

For drought stress, 40-day-old WT and T_1 transgenic plants were transferred into a nutrient solution containing 20% (W/V) PEG 6000. Osmotic potential of the solution was −1.01 MPa measured with a freezing point osmometer. The osmotic potential without PEG was −0.02 MPa. The concentration of PEG was maintained daily by changing the nutrient solution.

RT-PCR analysis of transformants

Transformed rice plants (T_1) were analyzed for the presence of *pMnSOD* transcripts after 10 h with 20% PEG treatment. Total RNA were prepared using RNAex Reagent & Systems (Watson Biotech, Shanghai, China) from leaves of WT and T_1 plants with and without PEG treatment. RT-PCR was performed using an mRNA Selective PCR Kit (TaKaRa Biotech, Dalian, China). One microgram of total RNA was primed with oligo(dT)$_{14}$ and reverse transcribed in a total volume of 25 μL. Five μL of reverse transcription products were used for PCR with primers P3 (5′-CCC CAA TTC TCA ACC CTT-3′) and P4 (5′-TTC TCA TAT ACT TCA CTG GCA-3′), which was located in the TP and *pMnSOD* sequence, respectively (Fig. 1). To ensure uniform amounts of template, PCR reactions were performed simultaneously with ubiquitin (UBI) primers (Nishi et al., 1993). The PCR was performed for 25 cycles and 12 μL of PCR products were checked on 1% agarose gel.

SOD activity assay

After 48 h with 20% PEG treatment, total SOD activity was assayed by measuring the ability of leaf homogenate to inhibit the reduction of nitro blue tetrazolium chloride (NBT) at 560 nm (Giannopolitis and Ries, 1977). One unit of SOD activity causes a half-maximal inhibition of the rate of light-induced, riboflavin-mediated reduction of NBT. The total SOD activity of leaf tissue under normal conditions was measured as the control. Protein concentration was determined by the Coomassie Brilliant Blue method (Bradford, 1976), using bovine serum albumin (BSA) as standard.

Ion leakage analysis

The oxidative stress tolerance of the plants was assessed by ion leakage analysis as described by Bowler et al. (1991) with some modifications. Leaves from WT and T_1 plants were cut into slices (1 cm in length) after 48 h with 20% PEG induction and floated on 0.4 M sorbitol or with an additional 5 μM of MV. Leaf slices were incubated overnight in the dark followed by illumination at 150 μmol/m^2/s light intensity for 18 h at 25 °C. The MV-induced oxygen radical damage was estimated by ion

leakage from the leaf slices measured using a conductivity meter. The relative ion leakage of leaf slices from plants under normal conditions was also measured as the control.

Photosynthesis assay

The CO_2 fixation was measured with a portable CO_2 gas analyzer (model CI-301; CID Inc., USA). Before and after 20% PEG treatment, net CO_2 assimilation rate was measured every hour from 7:00 to 19:00 measuring the second leaf from the tip under the following conditions: 200 μmol/m^2/s light intensity, 320 ppm CO_2, 28 °C and 70% relative humidity. The relative net photosynthesis rate (RP) was calculated by the following formula:

$$RP(\%) = (P_n/P_0) \times 100,$$

where P_n is the average net RP on a specific day (average hourly net photosynthetic rates of that day to get P_n) and P_0 is the average net RP before PEG treatment.

Statistical analysis

The randomized complete block design with blocks down in space was used in SOD activity, ion leakage, and photosynthesis assay. Means $\pm$SE were calculated from the data of three replications. Assays for different treatments were done at the same time. Differences between treatment means were determined by a *T*-test at $P \leqslant 0.05$.

Results

Expression of pMnSOD in transgenic rice plants upon drought stress

Fifteen independent lines of transgenic rice plants were produced by transforming *Oryza sativa* with pCAMBIA1301-*pMnSOD* using *Agrobacterium*-mediated transformation. There were no apparent phenotypic differences between the transgenic plants and WT plants. All of the transgenic lines were fertile and the T_1 progeny from two lines were used for further study. The 3:1 segregation ratio of GUS staining in T_1 plants suggested that the gene was integrated into the rice genome at a single locus. We used PCR to confirm the presence of the *pMnSOD* gene in rice. The expected 1455 bp band was amplified from T_0 transgenic plants using the P1 and P2 primers (Fig. 2). The PCR products were sequenced and they matched the expected sequence.

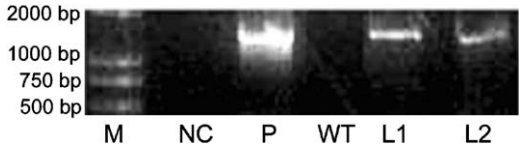

Figure 2. Genomic PCR analysis of T_0 transgenic rice plants. Genomic DNA was used as template for PCR using P1/P2 as the primers. The expected size of the product was 1455 bp. M, DNA marker; NC, negative control (water); P, plasmid pCAMBIA1301-pMnSOD; WT, wild type plant; L1, transgenic plant line 1; L2, transgenic plant line 2.

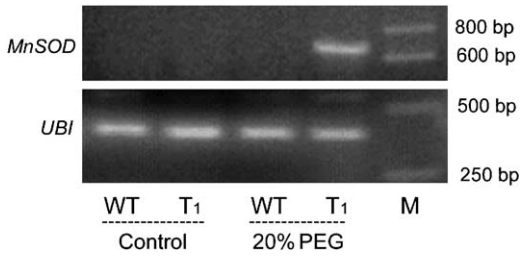

Figure 3. RT-PCR analysis of MnSOD expression in T_1 transgenic plants after 10 h with 20% PEG treatment. The cDNA was amplified with P3/P4 as primers, and the expected size of pMnSOD product was 671 bp. UBI with an expected size 396 bp was amplified simultaneously to ensure uniform amounts of templates.

In this study we use an oxidative stress-inducible *SWPA2* promoter (Kim et al., 2003) to control the expression of *pMnSOD* under stress conditions. The *SWPA2* promoter can be induced in transgenic tobacco plants by wounding, hydrogen peroxide and UV illumination. Levels of expresssion ranged from 100% to 150% of those of the CaMV 35S promoter under the same stress conditions measured by the activity of GUS reporter enzyme, whereas its transcripts—peroxidase mRNA were not detected in intact sweet potato plants (Kim et al., 1999, 2003). Here we used RT-PCR to confirm that this promoter can also be induced under drought stress in transgenic rice. A 671 bp fragment was amplified from transgenic rice after 10 h with 20% PEG treatment as expected after amplification with the P3 and P4 as primers (Fig. 3). Exogenous *pMnSOD* transcripts were not expressed in T_1 plants under normal conditions.

We also measured the total SOD activity in WT and T_1 leaves of plant. There were no differences in SOD activity between T_1 and WT plants in normal conditions, whereas after 48 h with 20% PEG treatment the total SOD activity in T_1 plants was about 1.4-fold higher than in WT plants (Fig. 4). Our data suggested that the *pMnSOD* gene expression

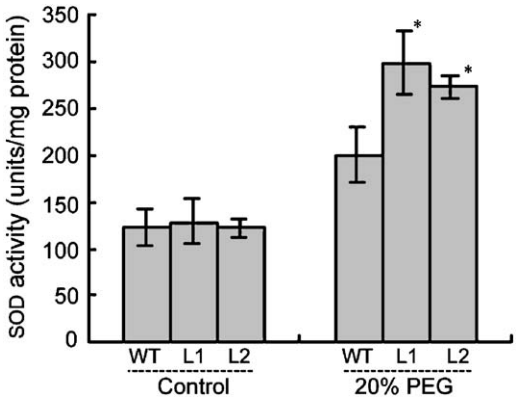

Figure 4. Total SOD activity (units/mg protein) in WT and T_1 transgenic plants under normal conditions and after 48 h 20% PEG treatment. L1 and L2 represent line 1 and line 2 of T_1 transgenic plants, respectively. Vertical bars represent standard errors ($n = 3$ per experiment). Values significantly different from the WT in the same group according to *T*-test are indicated by asterisks (*, $P \leqslant 0.05$).

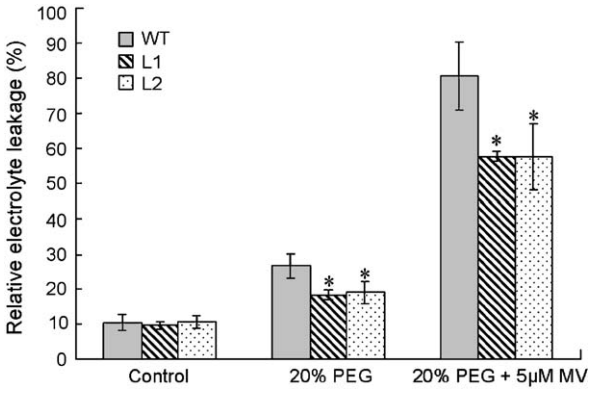

Figure 5. Protection against membrane damage in T_1 transgenic plants. The relative electrolyte leakage of WT and transgenic plants were measured under three conditions: normal growth conditions, control; pre-treatment with 20% PEG for 48 h, 20% PEG; pre-treatment with 20% PEG for 48 h followed by 5 μM MV treatment under illumination, 20% PEG+5 μM MV. Plant lines are as indicated in Fig. 4. Vertical bars represent standard errors ($n = 3$ per experiment). Asterisk (*) means values significantly different from the WT in the same group according to *T*-test ($P \leqslant 0.05$).

under drought stress contributed to a higher SOD activity in T_1 plants.

Protection from membrane damage in transgenic plants under drought stress

Disruption of the membrane integrity by stress can be estimated by measuring the leakage of cytoplasmic solutes from leaf slices. Under normal conditions there were no differences in electrolyte leakage between WT and T_1 plants (Fig. 5). After 48 h with 20% PEG treatment the ion leakage increased about 2.6- and 1.7-fold in WT and T_1 plants, respectively (Fig. 5). This data suggested that membranes of T_1 plants were injured less by the drought stress treatment than WT. Membrane damage is caused mainly by ROS generated in stress conditions. The significant difference in relative ion leakage between WT and T_1 plant was maintained when the plants were challenged with severe oxidative stresses, such as 5 μM MV after 20% PEG pre-treatment. Their relative ion leakage increased about 7.8- and 5.2-fold in WT and T_1 plants, respectively, compared to control plants in normal conditions (Fig. 5).

The effect of light mediated MV treatment on leaf discs has been used to test the tolerance to oxidative stress (Bowler et al., 1991; Yun et al., 2000). When illuminated, chloroplasts accept an electron from PSI, which is then donated O_2 creating O_2^-, while, MV treatment in vitro causes chlorophyll damage and leaf bleaching due to O_2^-. We observed that the degree of leaf bleaching was more severe in WT than in T_1 transgenic plants pre-treated with PEG (data not shown). This result suggests that expression of the *pMnSOD* upon PEG treatment in T_1 plants could protect chloroplasts from oxidative damage.

Effect of pMnSOD in protecting photosynthesis under drought stress

In order to analyze whether pMnSOD expression in chloroplasts can prevent damage to the photosynthesis apparatus we measured the net photosynthetic rate of WT and transgenic plants. In the first day after PEG treatment, the average photosynthetic rate in WT plants decreased to 67% of the initial rate whereas it was maintained at 92% and 80% in two transgenic lines, respectively (Fig. 6). In the two following days the photosynthetic rate recovered slowly in both WT and T_1 plants, reaching 74% of the initial level in WT, and 101% and 95% in the two transgenic lines ultimately. This suggested that photosynthesis was less affected by drought and recovered more rapidly in transgenic *pMnSOD* rice than in WT plants.

Discussion

In this study we characterized transgenic rice plants expressing pea *MnSOD* localized to the chloroplast. No growth retardation was observed in the transgenic plants growing in normal conditions. RT-PCR analysis and a SOD activity assay showed that the *pMnSOD* transgene was induced by

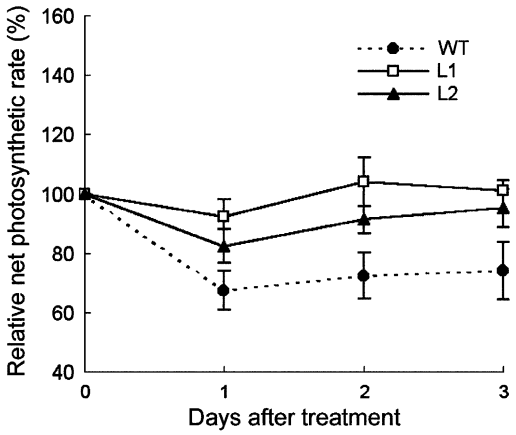

Figure 6. Effect of transgenic MnSOD on photosynthesis under 20% PEG treatment. Data are expressed as a percentage of the pre-stressed net photosynthetic rate. PEG was added at 21:00 on day 0. Vertical bars represent standard errors ($n = 3$ per experiment). At days 1–3, values of L1 and L2 were significantly different from that of WT according to *T*-test ($P \leqslant 0.05$).

drought stress mediated by PEG, whereas under normal conditions its expression was almost undetectable. That occurred because the *pMnSOD* expression is under the control of an oxidative stress-inducible *SWPA2* promoter, not the strong constitutive promoter, such as CaMV 35S promoter. Lack of *pMnSOD* expression in normal growth conditions can avoid possible negative side effects caused by over accumulation of the transgene products (Kasuga et al., 1999; Yoshida and Shinmyo, 2000). To our knowledge this is the first time an inducible promoter has been used in SOD transformation experiments. Using the *SWPA2* promoter also made it possible to switch on the expression of *pMnSOD* at the same time as the oxidative stress treatments were applied.

The toxic effects of many environmental stresses are partially due to generation of ROS. ROS can attack cellular macromolecules, generate lesions in DNA, cause membrane damage, and affect protein synthesis and stability. The response of stomata to osmotic stress is closure triggered by ABA that limits CO_2 availability, resulting in decreased photosynthesis (Osmond and Grace, 1995) and increased ROS generation, which gives rise to an oxidative environment in the cells (Asada, 1994). It has been reported that generation of superoxide is promoted in drought stressed plant cells because of impaired electron transport in the chloroplasts due in part to the accumulation of high concentrations of iron (Price et al., 1989). Superoxide can lead to the formation of hydroxyl radicals (Puppo and Halliwell, 1988), and that causes the primary symptoms observed in plants damaged by dehydration. In our study we measured ion leakage, which reflects membrane damage, observing that it increased after 20% PEG treatment in both WT and T_1 plants but to a significantly lesser extent in T_1 (Fig. 5). The membrane damage is partially caused by oxidative stress, a secondary effect of drought stress. The reduction in membrane damage in T_1 plants is probably due to increased SOD activity after PEG treatment (Fig. 4), which provides higher ROS scavenging efficiency. This protective effect was maintained when the T_1 plants were challenged with severe oxidative stress induced by MV after pre-treatment with PEG (Fig. 5).

The current literature does not provide a clear picture of the extent of *SOD* over-expression required to protect plants from oxidative stress. Tepperman and Dunsmuir (1990) demonstrated that a 30- to 50-fold increase in chloroplastic SOD activity of transgenic tobacco plants transformed with petunia *Cu/ZnSOD* failed to provide any detectable changes in oxidative stress resistance. Controversial results were obtained from transgenic tobacco plants transformed with pea *Cu/ZnSOD*, where expression of the transgene resulted in a 3-fold increase in total SOD activity (Sen Gupta et al., 1993b) and a significant increase in resistance to MV-induced membrane damage. Basu et al. (2001) also showed that a 1.5- to 2.5-fold increase in total SOD activity in transgenic *Brassica napus* plants transformed with wheat *MnSOD* increased oxidative resistance compared with the WT controls. In our study, an increase of about 1.4-fold in total SOD activity in the *pMnSOD* transgenic rice plants was enough to increase oxidative stress resistance and drought tolerance. Positive response to drought occurred as a consequence of over-expressing a single transgenic MnSOD targeted to chloroplasts in alfalfa (McKersie et al., 1996), an experiment perhaps comparable to ours. Chloroplasts are major sites for ROS production and therefore especially sensitive to ROS damage (Foyer et al., 1994). We fused the *pMnSOD* gene with a chloroplast transit peptide sequence in order to target the pMnSOD to the chloroplast. This may be one of the reasons why a relatively small increase in total SOD activity in T_1 plants provided a significant protective effect against drought stress (Fig. 5). Similarly, constitutively overexpressing *MnSOD* in transgenic tobacco chloroplasts and mitochondria resulted in reduced cellular damage by superoxide (Bowler et al., 1991). Over-expressing *Arabidopsis* FeSOD in transgenic maize chloroplast also enhanced tolerance toward MV and the MV tolerance correlated well with the transgenic FeSOD activity (Van Breusegem et al., 1999).

Enhanced drought tolerance of T_1 transgenic plants was associated with increased net

photosynthetic rate after PEG treatment (Fig. 6). The reduction of photosynthesis under drought stress may be caused by many factors, among which ROS should be considered. In chloroplasts, thylakoid-bound and stromal ROS scavenging enzymes include SOD, ascorbate peroxidase (APX), and several other enzymes such as monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) that are involved in the water–water cycle. They function by scavenging ROS and dissipating excess photons (Asada, 1999). The primary function of the water–water cycle is to protect chloroplastic components from ROS, which must be completely scavenged to preserve photosynthetic activity. If ROS are not scavenged effectively, oxidation of target molecules inactivates CO_2 fixation through effects on Calvin cycle enzymes including rubisco, photorespiration enzymes such as glutamate synthase (Kozaki and Takeba, 1996), the water–water cycle (APX and SOD), and PS I activity itself. These effects together lower the photon-using capacity of chloroplasts and further enhance the inhibition of photosynthesis. The de novo synthesis system of D1 protein of PS II in chloroplasts for repairing PS II photoinhibition is also sensitive to ROS (Nishiyama et al., 2000), and SOD can protect PS II from superoxide generated by oxidative and water stress (Martinez et al., 2001). Our data also suggest that higher expression SOD can protect the photosynthetic apparatus via protecting the relevant proteins and lipids, as transgenic plants showed higher net photosynthetic rate than WT controls under drought stress.

In conclusion, we developed transgenic rice plants expressing MnSOD in chloroplasts under the control of a stress inducible promoter. The transgenic plants had normal growth habit and appearance when grown under normal conditions. Our results indicate that ROS are involved, to some degree, in the reduction of photosynthetic capacity of rice during drought stress and that transgenic plants showed enhanced drought tolerance via the increased antioxidant capacity from the higher SOD activity.

Acknowledgments

This work was a Sino-Korea joint project supported by the Key Basic Research Special Fund of CAS (KSCXZ-SW-116), Key Basic Research Special Fund of CAS (KSCXZ-SW-116) and The National Key Basic Research Special Fund of China (G1999011700). We thank Jin-Ting Cheng for her help with the *Agrobacterium* transformation and Ya-Fang Zhu for her maintenance of the plants. We also thank Dr. David Lane for correction of the English in the manuscript, and Dr. Xin Yu for his helpful suggestions on primer design.

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