Enhancement of Oxidative Stress Tolerance in Transgenic Tobacco Plants Overproducing Fe-Superoxide Dismutase in Chloroplasts

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A chimeric gene consisting of the coding sequence for chloroplastic Fe superoxide dismutase (FeSOD) from Arabidopsis thaliana, coupled to the chloroplast targeting sequence from the pea ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit, was expressed in Nicotiana tabacum cv Petit Havana SR1. Expression of the transgenic FeSOD protected both the plasmalemma and photosystem II against superoxide generated during illumination of leaf discs impregnated with methyl viologen. By contrast, overproduction of a mitochondrial MnSOD from Nicotiana plumagoindiolosa in the chloroplasts of cv SR1 protected only the plasmalemma, but not photosystem II, against methyl viologen (L. Slooten, K. Capiau, W. Van Camp, M. Van Montagu, C. Sysaema, D. Inzé [1995] Plant Physiol 107: 737–750). The difference in effectiveness correlates with different membrane affinities of the transgenic FeSOD and MnSOD. Overproduction of FeSOD does not confer tolerance to H₂O₂, singlet oxygen, chilling-induced photoinhibition in leaf disc assays, or to salt stress at the whole plant level. In nontransgenic plants, salt stress led to a 2- to 3-fold increase in activity, on a protein basis, of FeSOD, cytosolic and chloroplastic Cu/ZnSOD, ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase. In FeSOD-overproducing plants under salt stress, the induction of cytosolic and chloroplastic Cu/ZnSOD was suppressed, whereas induction of a water-soluble chloroplastic ascorbate peroxidase isozyme was promoted.

The photosynthetic electron transport chain contains at the acceptor side of PSI a number of autoxidizable enzymes that reduce oxygen to superoxide (reviewed by Badger, 1985; Asada and Takahashi, 1987; Asada, 1994). These include Fd (Purbank and Badger, 1982; Hosein and Palmer, 1983) and the Fe-S centers X or A/B in the aprotic membrane interior of the PSI reaction center complex (Takahashi and Asada, 1988). The superoxide may mediate cyclic electron flow around PSI (Asada, 1994) or it may diffuse it to the stromal membrane surface, where it is dismutated to oxygen and H₂O₂ in nonenzymic and enzymic reactions (see below). Recent evidence suggests that superoxide and H₂O₂ can also be produced by PSII during high-light treatment of thylakoids or intact chloroplasts (Landgraf et al., 1995). H₂O₂ at 10 μM inhibits CO₂ fixation (Kaiser, 1979); at concentrations as low as 1 μM it causes a substantial inactivation of thiol-modulated Calvin cycle enzymes (Buchanan, 1980, 1991; Takeda et al., 1995). Superoxide can inactivate some metal-containing enzymes such as the Fd-linked nitrate reductase, catalase, and peroxides (for review, see Asada and Takahashi, 1987). But the chief danger is that H₂O₂ can react with reduced metal ions, especially Fe, resulting in the formation of the hydroxyl radical. The hydroxyl radical initiates self-propagating reactions leading to peroxidation of membrane lipids, base mutations, breakage of DNA strands, and destruction of proteins (Asada and Takahashi, 1987; Halliwell, 1987; Bowler et al., 1992). In addition, there is some evidence that part of the superoxide generated in illuminated chloroplasts diffuses toward the thylakoid lumen. Because of the low pH in the lumen during illumination, the superoxide can be protonated in that compartment, yielding the perhydroxyl radical, which, unlike the superoxide anion, can initiate lipid peroxidation directly (for review, see Asada and Takahashi, 1987). The conditions leading to damage caused by active oxygen species will be referred to as oxidative stress.

Formation of active oxygen species in the chloroplasts is enhanced when carbon assimilation is inhibited. Oxidative...
stress arises, for example, when high irradiance is combined with chilling temperatures, drought, or heat (Bowler et al., 1992). Salt stress is another condition that may give rise to oxidative stress, as suggested by the increase in activities of antioxidant enzymes in response to high salinity, and by the correlation of salt tolerance with antioxidant enzyme levels (Gossett et al., 1994; Olmos et al., 1994; Hernandez et al., 1995; Sehmer et al., 1995).

An elaborate antioxidant system, a defense against oxidative stress, is present in the chloroplasts. SOD catalyzes the dismutation of superoxide into oxygen and H₂O₂. SODs are classified, according to their metal cofactor, as FeSOD, MnSOD, or Cu/ZnSOD. Chloroplasts generally contain Cu/ZnSOD and, in a number of plant species, FeSOD (Van Camp et al., 1994a). APx reduces H₂O₂ to water, with ascorbate as the electron donor. Chloroplastic APx was recently found to occur in a stromal and a membrane-bound form (Miyake and Asada, 1992). The re-reduction of the reaction product of APx, monodehydroascorbate, proceeds along different pathways, depending on the type of APx involved. The reaction product of stromal APx is reduced by NADPH, either directly or via glutathione. The enzymes taking part in this so-called ascorbate-glutathione cycle (Foyer and Halliwell, 1976; Nakano and Asada, 1981) are GR, DHAR, and MDHAR (for reviews, see Asada and Takahashi, 1987; Halliwell, 1991). APx was recently found to occur in a stromal and a membrane-bound form (Miyake and Asada, 1992). The re-reduction of the reaction product of APx, monodehydroascorbate, proceeds along different pathways, depending on the type of APx involved. The reaction product of stromal APx is reduced by NADPH, either directly or via glutathione. The enzymes taking part in this so-called ascorbate-glutathione cycle (Foyer and Halliwell, 1976; Nakano and Asada, 1981) are GR, DHAR, and MDHAR (for reviews, see Asada and Takahashi, 1987; Halliwell, 1987). In contrast, monodehydroascorbate produced by membrane-bound APx is re-reduced directly by Fd (Miyake and Asada, 1994).

The overproduction of antioxidant enzymes provides a way to study the role of these enzymes in the antioxidant system, and to study the contribution of oxidative stress tolerance to tolerance to physiological types of stress. Overproduction of SOD in the chloroplasts has been found to result in enhanced oxidative stress tolerance in transgenic tobacco (Bowler et al., 1991; Sen Gupta et al., 1993a; Foyer et al., 1994; Van Camp et al., 1994b; Slooten et al., 1995), alfalfa (McKersie et al., 1993), potato (Perl et al., 1994), and, according to preliminary data, in cotton (Allen, 1995). Similar results were obtained with overproduction of SOD in the mitochondria of alfalfa (McKersie et al., 1993) and in the cytosol of potato (Perl et al., 1993). In many of these studies, oxidative stress tolerance was assessed in assays based on the use of MV. This herbicide passes electrons from various electron transport chains to oxygen, generating superoxide. During illumination, MV generates superoxide primarily in the chloroplasts (Halliwell, 1984; Slooten et al., 1995) and thus simulates the oxidative stress component of the environmental stresses. In addition, an enhanced tolerance to freezing stress in transgenic alfalfa overproducing SOD in the chloroplasts has been reported (McKersie et al., 1993), and transgenic tobacco overproducing SOD in the chloroplasts exhibits an enhanced tolerance to chilling in the dark (Foyer et al., 1994) or in the light (Sen Gupta et al., 1993a).

We have shown that expression of plant mitochondrial MnSOD in the chloroplasts of transgenic Nicotiana tabacum cv SR1 and cv PB6 reduces cellular damage generated by treatment with MV (Bowler et al., 1991; Slooten et al., 1995) or ozone (Van Camp et al., 1994b). In PB6, overproduction of MnSOD had a clear protective effect on MV-induced ion leakage and, albeit to a lesser extent, also on MV-induced inactivation of the PSII reaction center. In SR1, protection was observed with regard to ion leakage, but not with regard to the PSII reaction center (Slooten et al., 1995). Apparently, improvement in the antioxidant defense was more pronounced at the plasmalemma than at PSII, in spite of the fact that the overproduced SOD is located in the chloroplasts. It is possible that the pathway from MV-mediated superoxide generation at PSI to damage at PSII is poorly accessible to plant mitochondrial MnSOD expressed in the chloroplasts. This lack of protection of PSII might be specific for overproduced plant mitochondrial MnSOD, since chloroplastic overproduction of Cu/ZnSOD in potato (Perl et al., 1993) and of Escherichia coli MnSOD in tobacco (Foyer et al., 1994) does alleviate the inhibition of photosynthesis by MV.

Here we report that overproduction of FeSOD in the chloroplasts of N. tabacum cv SR1 protects both the plasmalemma and PSII from MV-induced damage. This demonstrates that FeSOD provides better protection of chloroplasts than MnSOD. We argue that this is because FeSOD, in contrast to MnSOD, is indigenous to the chloroplasts. Functional differences between the FeSOD and MnSOD enzymes of E. coli have been reported with regard to protection of DNA and proteins (Hopkin et al., 1992). The data presented in this study, to our knowledge, are the first to demonstrate that in plant chloroplasts, FeSOD and MnSOD have different protective properties that may be related to their suborganellar location. We also show that overproduction of FeSOD interferes with signal pathways, leading to induction of cytosolic Cu/ZnSOD during salt stress. In addition, overproduction of FeSOD leads to induction of chloroplastic APx during salt stress.

**MATERIALS AND METHODS**

**Generation of FeSOD-Overproducing Plants**

To construct pEXSOD10, a PCR product was generated with the 5′ primer TCAAGTGCTGTAGATCTAACAGCCTAC and the 3′ primer TCACTCAGAAAAG (complementary to positions 842-871 in the FeSOD gene). The amplified fragment was cloned in a pCM2 vector, HincII-digested pUC18. The fragment was re-excised by a BamHI-BglII digest and cloned into the ClaI-BamHI-digested binary vector pGSJ780A to construct pEXSOD10.
liflower mosaic virus 35S promoter. Transformation of *Nicotiana tabacum* var Petit Havana SR1 with this cassette, which was named pEXSOD10, was according to the methods of De Block et al. (1987).

**Plant Material**

Plants were grown in pots on peat-based compost containing fertilizer and were watered with demineralized water to which, after bolting, 1 vol % of a commercial fertilizer (NPK 6-3-6) was added. Unless otherwise indicated, the plants were grown in a 12-h light/12-h dark cycle, with day and night temperatures of about 22 and 16°C, respectively. The light, provided by mercury-halogen vapor lamps with a daylight spectrum (HQI-T, Osram, Munich, Germany), had an intensity of approximately 135 µmol m⁻² s⁻¹. Unless indicated otherwise, the experiments were carried out on the seventh to ninth leaves from the top at 10 to 12 weeks after sowing.

In some experiments the plants were transferred to a growth chamber at 1 month after sowing (i.e. at the fifth-leaf stage), at which time they were grown in a 12-h light/12-h dark cycle at 22/15°C. In the course of 8 d the light intensity was gradually raised from 180 to 650 µmol m⁻² s⁻¹. The light intensity remained at this level during the next 14 d, and the experiments were performed at the end of this period.

In the salt-stress experiments, plants were grown at an intensity of 65 µmol m⁻² s⁻¹, with day and night temperatures of about 25 and 20°C, respectively. The plants were watered with demineralized water, to which 60 mM NaCl was added from d 12 after sowing.

**Assessment of Oxidative Stress Tolerance**

Leaf discs of approximately 1.5 cm² were preincubated overnight with reagents that mediate the formation of toxic oxygen species during subsequent illumination of the leaf discs: MV generates superoxide (Bowler et al., 1991), AT generates H₂O₂ (see “Results”), and eosin generates singlet oxygen (Knox and Dodge, 1985). The MV assays were carried out as described by Slooten et al. (1995). In the case of AT or eosin treatment, the leaf discs were placed in Petri dishes containing 3 mL of aqueous solutions of these compounds. The Petri dishes were put in a gas-tight, thermostatted container with a glass lid. The leaf discs were illuminated with white light from mercury-halogen vapor lamps, filtered by 8 cm of water. The light intensity was 360 µmol m⁻² s⁻¹. The temperature in the Petri dishes during illumination was maintained at 18°C. After illumination the leaf discs were incubated in the dark for at least 2 h to ensure complete dark adaptation prior to measuring F₆/F₉max, as a measure of the activity of the PSII reaction centers. This ratio gives the exciton trapping efficiency when all photochemical traps are open (Genty et al., 1989). The fluorescence measurements were made as described by Slooten et al. (1995). The conductance of the floating solutions was determined as a measure of ion leakage from the leaf discs, which was due to lipid peroxidation of the cell membranes (Slooten et al., 1995).

For the photoinhibition experiments, the leaf discs were floated overnight on an aqueous solution of 75 µM of the chloroplast translation inhibitor chloramphenicol prior to illumination. At this concentration chloramphenicol does not act as a PSI electron acceptor (Okada et al., 1991).

**Biochemical Assays**

The basal medium for the preparation of leaf disc extracts for enzyme assays contained 50 mM potassium phosphate, 0.1% Triton X-100, 20% (w/v) Suc, 2% polyvinylpolypyrrolidone, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 4 mM benzamidine, 4 mM caproic acid, and KOH to pH 7.6. Suc and polyvinylpolypyrrolidone were omitted from the basal medium used for the preparation of extracts for SOD determinations. Whole-leaf extracts were prepared by grinding leaf discs (0.2 g) in an ice-cold mortar with 1.2 mL of basal medium supplemented with the following additions: for APx, GR, and MDHAR, 30 mM sodium ascorbate; for GA3P-DH, 30 mM sodium ascorbate and 0.05% (w/v) β-mercaptoethanol; for DHAR, 2 mM sodium ascorbate; and for SOD, 0.05% (w/v) β-mercaptoethanol and 0.1 mg/mL BSA. The extracts were centrifuged for 30 min at 40,000 g. The supernatants were stored on ice and used the same day. The assays of APx, DHAR, GR, and MDHAR were carried out as described previously (Slooten et al., 1995). GA3P-DH was assayed as described by Stitt et al. (1989). For these assays, 1 unit equals 1 µmol of substrate converted per min.

APx activity on non-denaturing polyacrylamide gels was detected as described by Mittler and Zilinskas (1993). SOD activity was determined first in a solution assay, and then after an activity staining following electrophoresis on non-denaturing polyacrylamide gels (Bowler et al., 1991). The gels were stained as described previously (Slooten et al., 1995) and then scanned with a one-dimensional scanning densitometer. The relative contribution of each of the SOD isoforms to the overall activity was determined from the contribution of the area under the corresponding peak of the densitogram to the total area. The overall SOD activity in leaf disc extracts was determined in the solution assay with the same assay mixture. In the solution assay one unit of SOD activity causes a half-maximal inhibition of the rate of light-induced, riboflavin-mediated reduction of nitroblue tetrazolium. In both assays the activity was measured with and without 2 mM KCN, which causes a virtually complete inhibition of Cu/ZnSOD (Geller and Waring, 1984).

For the preparation and washing of chloroplasts we used the low-salt grinding and resuspension media described by Cerovic and Plesnicar (1984), except that β-mercaptoethanol was added to both media at 0.05% (v/v). Chloroplasts were prepared by grinding 4 g of leaf material for 3 s in 40 mL of ice-cold grinding medium in a blender (Waring). The homogenate was filtered through four layers of Miracloth (Calbiochem) and centrifuged for 1.5 min at 600 g at 4°C. The sedimented chloroplasts were washed twice in 20 mL of resuspension medium, followed by centrifugation as before.
Chlorophyll in whole-leaf extracts was determined as described previously (Slooten et al., 1995). Protein was estimated according to Bradford (1976) using BSA as a standard. All absorbance measurements were carried out on a spectrophotometer (Cary 2300, Varian Techtron, Mulgrave, Victoria, Australia).

RESULTS

SOD Activity Levels in Nonstressed Transgenic and Control Plants

To overexpress chloroplastic FeSOD from A. thaliana in tobacco chloroplasts, we made a chimeric gene in which the FeSOD coding region was fused in frame with the coding sequence for the chloroplast transit peptide of the Rubisco small subunit. The expression of this gene fusion was driven by the 35S promoter from the cauliflower mosaic virus. Relatively high levels of FeSOD activity were observed in leaf extracts of seven primary transformants. Six of these lines, each containing a single copy of the transgene, were made homozygous and selected for further study. Most of the data shown below were obtained with line SR1-14, but the results were confirmed with several other lines.

Figure 1 shows the banding patterns observed after non-denaturing electrophoresis of leaf extracts or chloroplasts from mature leaves, followed by SOD activity staining. The bands are numbered in order of increasing mobility. In leaf extracts from nontransgenic plants (Fig. 1A, dashed line), three bands were observed after staining without KCN: band 3 contains both chloroplastic FeSOD and cytosolic Cu/ZnSOD, and bands 4 and 5 represent chloroplastic Cu/ZnSOD (Slooten et al., 1995). Preincubation with KCN prior to staining caused a complete inactivation of Cu/ZnSOD; the remaining activity represents only FeSOD (Fig. 1B, dashed line, band 3'). In chloroplast extracts from nontransgenic plants (Fig. 1C, dashed line), cytosolic Cu/ZnSOD was absent, and band 3' again corresponds to chloroplastic FeSOD. Pretreatment with KCN abolished the chloroplastic Cu/ZnSOD activity, but had little effect on the activity of chloroplastic FeSOD (Fig. 1D, dashed line). Extracts from immature leaves also contain a small, slow-moving band corresponding to mitochondrial MnSOD (not shown) (Slooten et al., 1995).

Leaf and chloroplast extracts from FeSOD-overproducing plants (line SR1–14) exhibited two extra bands (nos. 1 and 2) representing trFeSOD (Fig. 1, solid lines). Similar results were obtained with other transgenic lines (not shown). In the KCN-treated lanes, the endogenous FeSOD shows up as a small shoulder at the fast-moving side of band 2 (Fig. 1, B and D, solid lines). A comparison with the corresponding traces from nontransgenic plants indicates that FeSOD-overproducing plants contained about one-half of the endogenous FeSOD activity that was found in nontransgenic plants. Thus, either trFeSOD leads to a suppression of the endogenous FeSOD activity, or some of the endogenous FeSOD forms heterodimers with trFeSOD.

We used the SOD activity gels to estimate the activities of the different SOD isoforms, as described in "Materials and Methods." The staining intensities of the bands on the activity gels indicate that KCN, which was used to inactivate Cu/ZnSOD, also caused an inhibition of both endogenous and overproduced FeSOD. This can be seen most clearly in extracts prepared from isolated chloroplasts, which do not contain cytosolic Cu/ZnSOD (Fig. 1, C and D). The KCN inhibition amounted to 20 to 30% for both endogenous and overproduced FeSOD. In the gels without KCN, the contribution of cytosolic Cu/ZnSOD to band 3 was obtained by subtracting the contribution of FeSOD. This contribution was estimated from the KCN-treated gels, taking the observed KCN inhibition by FeSOD into account. The results for line SR1–14 are shown in Table I. In leaf extracts from SR1–14, we found an average of 13.8 units/mg chlorophyll of FeSOD, most of which represents transgenic FeSOD (cf. Fig. 1). There was no significant difference in activity of mitochondrial MnSOD and cytosolic and chloroplastic Cu/ZnSOD between transgenic and control plants.

### Table I. SOD activities in leaf extracts from FeSOD-overproducing and control plants

<table>
<thead>
<tr>
<th>SOD Species</th>
<th>Activity (13 plants)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MnSOD</td>
<td>0.62 ± 0.20</td>
</tr>
<tr>
<td>FeSOD</td>
<td>1.88 ± 0.19</td>
</tr>
<tr>
<td>Chloroplastic Cu/ZnSOD</td>
<td>5.23 ± 1.33</td>
</tr>
<tr>
<td>Cytosolic Cu/ZnSOD</td>
<td>6.04 ± 0.64</td>
</tr>
<tr>
<td>SR1</td>
<td>0.52 ± 0.17</td>
</tr>
<tr>
<td>SR1-14</td>
<td>13.85 ± 0.63</td>
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<tr>
<td>- Mean ± SE</td>
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**Figure 1.** Densitogram of the SOD-banding pattern of leaf extracts (A and B) and chloroplasts (C and D) from FeSOD-overproducing plants of line SR1–14 (solid lines) and control plants (dashed lines). The gels were stained in the absence (A and C) or presence (B and D) of 2 mM KCN. Migration is from left to right. Bands 1 and 2, Engineered FeSOD; band 3, a mixture of cytosolic Cu/ZnSOD and endogenous FeSOD; band 3', endogenous FeSOD; bands 4 and 5, chloroplastic Cu/ZnSOD.
Table II. KCN-insensitive SOD activity in leaf extracts from different transgenic lines overproducing FeSOD

<table>
<thead>
<tr>
<th>Line</th>
<th>Activity (units/mg chlorophyll)</th>
</tr>
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<tbody>
<tr>
<td>SR1 (control)</td>
<td>2.88 ± 0.43</td>
</tr>
<tr>
<td>SR1-23-1</td>
<td>10.56 ± 0.71</td>
</tr>
<tr>
<td>SR1-36-7</td>
<td>10.84 ± 0.39</td>
</tr>
<tr>
<td>SR1-42-8</td>
<td>12.34 ± 0.73</td>
</tr>
<tr>
<td>SR1-14</td>
<td>14.37 ± 0.65*</td>
</tr>
<tr>
<td>SR1-32-2</td>
<td>17.21 ± 0.85</td>
</tr>
<tr>
<td>SR1-21-1</td>
<td>17.32 ± 1.04</td>
</tr>
</tbody>
</table>

*13 plants.

The data shown in Table I indicate that the total activity of chloroplastic SOD species (FeSOD and chloroplastic Cu/ZnSOD) was, on a chlorophyll basis, in line SR1-14 approximately 2.5 times higher than in control plants. However, chloroplastic Cu/ZnSOD is quite variable in activity; it occurs only in young, expanding leaves, and previous experiments yielded no indication that it provides tolerance against light-dependent, MV-mediated oxidative stress (Slooten et al., 1995). Therefore, the ratio of FeSOD contents may be more relevant in this respect. The chloroplastic FeSOD activity was, on a chlorophyll basis, in line SR1-14 approximately 7.4 times higher than in control plants.

The SOD activity observed in the presence of KCN represented mainly FeSOD (cf. Fig. 1), sometimes with a minor contribution by MnSOD (less than 10% of the total KCN-insensitive activity, as indicated by the banding patterns on the SOD activity gels). Table II shows the KCN-insensitive activities observed in the different transgenic lines. The difference in KCN-insensitive SOD activity between the highest and the lowest expressor amounted to only approximately 60%.

Oxidative Stress Tolerance in Transgenic and Control Plants

Plants overproducing FeSOD were clearly more tolerant to MV than control plants. This is shown in Figure 2 for young plants (9 weeks) and older plants (13 weeks) of line SR1-14. These plants were grown at a light intensity of 135 \( \mu \text{mol m}^{-2} \text{s}^{-1} \). Older plants are considerably more tolerant to MV than young plants, as indicated by the difference in scales along the x axis. We reported previously that in var SR1, overproduction of MnSOD provided protection against MV-induced ion leakage (due to cell membrane deterioration), but not against MV-induced inactivation of PSII (Slooten et al., 1995). In contrast, overproduction of FeSOD resulted in protection against both types of damage. This is indicated by the fact that the extent of MV-induced ion leakage (Fig. 2, A and B), as well as the extent of the MV-induced decrease in \( F_v/F_{\text{max}} \) (Fig. 2, C and D), were lower in transgenic plants than in control plants. Similar results were obtained with other transgenic lines (Fig. 3). Furthermore, similar results were obtained with plants grown at a light intensity of 650 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (Table III), although these plants were 15 to 30 times less sensitive to MV than plants grown at 135 \( \mu \text{mol m}^{-2} \text{s}^{-1} \).

AT inhibits catalase (Margoliash et al., 1960; Havir, 1992) and causes an increase in the \( \text{H}_2\text{O}_2 \) content of tobacco leaves during illumination (Chen et al., 1993); at the same time, it causes inactivation of endogenous, chloroplastic APx. Apparently as a consequence of the resulting increase in the concentration of \( \text{H}_2\text{O}_2 \) in the chloroplasts, the PSII reaction center is inactivated. This is not accompanied by any significant ion leakage from the leaf discs (L. Slooten, K. Capiau, S. Kushnir, M. Van Montagu, and D. Inzé, unpublished data). Plants overproducing FeSOD were not more tolerant to AT than control plants (Table IV).

Leaf discs impregnated with eosin generate singlet oxygen during illumination (Knox and Dodge, 1985). Plants overproducing FeSOD were not more tolerant to eosin than

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**Figure 2.** MV-induced conductance increase (A and B) and MV-induced decrease in \( F_v/F_{\text{max}} \) (expressed as a percentage of no MV) (C and D) in leaf discs from transgenic and control plants at 9 weeks (A and C) or 14 weeks (B and D) after sowing. Conductance increase was expressed in microsiemens cm\(^{-1}\) 35 mg\(^{-1}\) fresh weight. Error bars indicate SD for four plants. Open symbols, Control plants; solid symbols, SR1-14.
control plants. It made no difference in this respect whether the damage was assessed from the decrease in activity of the PSII reaction center (Table IV), or from the increase in the conductance of the floating solution (not shown).

In addition, plants overproducing FeSOD were not more tolerant to chilling-induced photoinhibition than control plants (Table V). It made no difference in this respect whether the photoinhibitory treatment was carried out for 4 h at 360 μmol m⁻² s⁻¹ or for 30 h at 30 μmol m⁻² s⁻¹.

Membrane Affinity of trFeSOD and trMnSOD

We reported previously that in var SR1, overproduction of MnSOD provided protection against MV-induced ion leakage (due to cell membrane deterioration), but not against MV-induced inactivation of PSII (Slooten et al., 1995). In contrast, overproduction of FeSOD resulted in protection against both types of damage. It seemed possible that this might be due to a different suborganellar location of the overproduced MnSOD and FeSOD. Specifically, these enzymes might have a different membrane affinity. To test this hypothesis, we determined the activity of the overproduced SODs and of a stromal marker enzyme, NADP-dependent GA3P-DH, in washed chloroplasts from MnSOD-overproducing plants and from FeSOD-overproducing plants. We made use of the observation that a considerable proportion of the chloroplasts became leaky during isolation and lost their stromal content before washing. One would expect that electrostatically membrane-associated enzymes would be washed out to a lesser extent than stromal enzymes. Prior to assay, the suspension was sonicated to the extent that the chloroplasts that until then had remained intact were disrupted in a medium in which the enzymes were quantitatively released. In addition, we measured the activity of GA3P-DH and of the overproduced SODs in leaf extracts prepared from the same leaf as the chloroplasts. All activities were expressed on a chlorophyll basis. For each enzyme, the recovery in the chloroplasts was calculated as the ratio of activities in sonicated chloroplasts to that in leaf extracts. The recovery of GA3P-DH was approximately 35%. We then calculated the ratio of recoveries of GA3P-DH over the overproduced SOD. This ratio is expected to be 1 for a stromal enzyme and lower than 1 for a membrane-associated enzyme. Finally, we calculated the ratio of recoveries of FeSOD and MnSOD (RFeSOD/RMnSOD) as the ratio between RGA3P-DH/RMnSOD in MnSOD-overproducing plants, and RGA3P-DH/RFeSOD in FeSOD-overproducing plants, where R is the recovery of the enzyme indicated in the subscript. This method allowed us to compare recoveries of FeSOD and MnSOD corrected for differences in quality of the chloroplast preparation. This procedure was repeated five times in independent experiments. The results are shown in Table VI. Overproduced MnSOD exhibited about the same recovery as GA3P-DH, indicating that it behaves like a stromal enzyme. Overproduced FeSOD exhibited higher recoveries, indicating electrostatic binding to chloroplast membranes. This was especially clear from the average ratio of recoveries of FeSOD and MnSOD, calculated as indicated above.

Effect of trFeSOD on Induction of Antioxidant Enzymes during Salt Stress

Plants respond to salt stress with increases in antioxidant enzyme activities, indicating that oxidative stress is involved in salt stress (Gossett et al., 1994; Hernandez et al., 1995). To study the effect of FeSOD overproduction on salt tolerance, we grew transgenic and control plants under salt stress (see “Materials and Methods.” Mean ± SE (number of plants).

| Table III. Enhancement of MV tolerance in FeSOD-overproducing plants |
| MV-induced conductance increase (in microsiemens cm⁻¹ 35 mg⁻¹ fresh weight) and MV-induced decrease in F/Fmax (in percentage of no MV) in leaf discs from transgenic and control plants grown at a light intensity of 650 μmol m⁻² s⁻¹. The MV concentration was 14 μM. Mean ± SE for six plants. |
| Plants | Conductance Increase | Decrease in F/Fmax |
| SR1-14 | 1.16 ± 0.49 | 10.12 ± 2.02 |
| SR1 | 4.02 ± 1.41 | 24.15 ± 2.02 |

| Table IV. Lack of enhancement of AT and eosin tolerance in FeSOD-overproducing plants |
| Leaf discs were illuminated for 16 h in the presence of 5 mM AT, or for 2 h in the presence of 0.1 mM eosin. For additional details, see “Materials and Methods.” Mean ± SE (number of plants). |
| Plants | Decrease in F/Fmax |
| | AT | Eosin |
| SR1 | 31 ± 1.4 (5) | 44.1 ± 2.5 (6) |
| SR1-14 | 30 ± 2.6 (5) | 47.7 ± 3.0 (6) |

Figure 3. MV-induced conductance increase (A), and MV-induced decrease in F/Fmax (expressed as a percentage of no MV) (B) in leaf discs from transgenic and control plants at 11 weeks after sowing. The MV concentration was 2 μM. Conductance increase was expressed in microsiemens cm⁻¹ 35 mg⁻¹ fresh weight. Error bars indicate SE for four plants. Numbers in the figures indicate the significance of the difference in mean values between transgenic and control plants, as established with a two-sided t test.
Salt-stressed plants accumulated considerably less biomass, in terms of fresh weight, than control plants (Fig. 4A). The salt concentration in the soil increased gradually with time, so that growth became increasingly inhibited (not shown). As a consequence, the ratio of fresh weights in salt-stressed plants over fresh weights in control plants decreased steadily with time (Fig. 4B). There was no difference in this respect between FeSOD-overproducing and control plants. In addition, there was no difference in dry matter accumulation between FeSOD-overproducing and control plants at the end of the experiment (Fig. 4C). At the end of the experiment, the concentration of dissolved sodium in the soil was 120 mM in the salt-treated pots, compared with 5 mM in the controls (not shown), and the sodium content of salt-stressed plants was approximately twice that of the control plants (Fig. 4D).

Salt stress was accompanied by oxidative stress, as indicated by increases in activities of all tested antioxidant enzymes in nontransgenic plants. Specifically, the activities of chloroplastic FeSOD, cytosolic Cu/ZnSOD, chloroplastic Cu/ZnSOD, APx, GR, and DHAR were approximately two to three times higher, on a protein basis, in salt-stressed nontransgenic plants than in unstressed nontransgenic plants at the end of the experiment (Fig. 4E). FeSOD-overproducing plants exhibited a similar increase in overall activity of APx, GR, and DHAR during salt stress. However the induction of cytosolic and chloroplastic Cu/ZnSOD, observed in control plants under salt stress, was suppressed in FeSOD-overproducing plants (Fig. 4E).

To discriminate between chloroplastic and cytosolic APx isozymes, we electrophoresed leaf extracts on non-denaturing polyacrylamide gels, and stained the gels for APx activity (Fig. 5). Three bands of APx activity can be distinguished after non-denaturing electrophoresis of leaf extracts from bolting tobacco plants: one band corresponding to cytosolic APx, and two bands corresponding to chloroplastic APx (L. Slooten, K. Capiau, S. Kusznir, M. Van Montagu, and D. Inzé, unpublished data). The latter two will be denoted as chlAPx-1 and chlAPx-2 for ease of reference. The plants used in the present experiments were still in the rosette stage, and were grown at a relatively low light intensity. Under those circumstances, only the cytosolic APx and chlAPx-2 were observed in extracts from unstressed, nontransgenic plants. Under salt stress, nontransgenic plants usually exhibited an increase in activity of chlAPx-2 in comparison with nonstressed plants. Unstressed, FeSOD-overproducing plants exhibited a similar isozyme pattern as unstressed nontransgenic plants. However, during salt stress, the FeSOD-overproducing plants exhibited an increase in activity of both chloroplastic APx isozymes. Thus, the induction of chlAPx-1 was accelerated specifically in FeSOD-overproducing plants under salt stress. 4-(Chloromercuri)benzenesulphonic acid, a specific inhibitor of ascorbate peroxidase (Chen

### Table V. Lack of enhancement of tolerance to chilling-induced photoinhibition in FeSOD-overproducing plants

Leaf discs floating on water containing 75 µM chloramphenicol were illuminated as indicated in closed Petri dishes. $F_{\text{r}}/F_{\text{max}}$ was measured 2 h after cessation of illumination. Mean ± se (number of plants).

<table>
<thead>
<tr>
<th>Light intensity</th>
<th>Duration</th>
<th>Temperature</th>
<th>SR1</th>
<th>SR1-14</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmol m⁻² s⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>360</td>
<td>4 h</td>
<td>7 °C</td>
<td>36.5 ± 1.5 (6)</td>
<td>39.1 ± 1.3 (6)</td>
</tr>
<tr>
<td>30</td>
<td>31 h</td>
<td>4 °C</td>
<td>24.8 ± 2.7 (4)</td>
<td>28.6 ± 2.1 (4)</td>
</tr>
</tbody>
</table>

### Table VI. Comparison of the recoveries of overproduced SODs in isolated chloroplasts

Washed chloroplasts were resuspended in 2.4 ml of the extraction medium used for determination of GA3P-DH in whole leaf extracts (see "Materials and Methods"), except that polyvinylpolypyrrolidone, Triton, and ascorbate were omitted. The chloroplasts were broken by sonication. Part of the supernatants were used for assay of SOD (spectrophotometric, as well as after nondenaturing PAGE) and of GA3P-DH. All data are presented as mean ± SD from five independent experiments. The activities of trtmSOD and trFeSOD in leaf extracts from transgenic plants were 11.8 ± 1.7 and 13.8 ± 2.3 units/mg chlorophyll, respectively. The activities of GA3P-DH in leaf extracts from MnSOD- and FeSOD-overproducing plants were 10.3 ± 0.9 and 10.4 ± 2.4 units/mg chlorophyll, respectively. R is the recovery in isolated chloroplasts of the enzyme indicated in the subscript. For other details, see text.

<table>
<thead>
<tr>
<th>MnSOD-Overproducing Plants</th>
<th>FeSOD-Overproducing Plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_{\text{MnSOD}}$</td>
<td>$R_{\text{FeSOD}}$</td>
</tr>
<tr>
<td>39.9 ± 14.8%</td>
<td>50.4 ± 18.4%</td>
</tr>
<tr>
<td>$R_{\text{CA3P-DH}}$</td>
<td>$R_{\text{CA3P-DH}}$</td>
</tr>
<tr>
<td>36.6 ± 11.4%</td>
<td>33.5 ± 18.8%</td>
</tr>
<tr>
<td>$R_{\text{CA3P-DH}}/R_{\text{MnSOD}}$</td>
<td>$R_{\text{CA3P-DH}}/R_{\text{FeSOD}}$</td>
</tr>
<tr>
<td>1.03 ± 0.41</td>
<td>0.69 ± 0.25</td>
</tr>
</tbody>
</table>

$R_{\text{FeSOD}}/R_{\text{MnSOD}} = 1.50 ± 0.12$
DISCUSSION

Several reports have been published on the overproduction of Cu/ZnSOD in plants (Tepperman and Dunsmuir, 1990; Pitcher et al., 1991; Perl et al., 1993; Sen Gupta et al., 1993a, 1993b) or MnSOD (Bowler et al., 1991; McKersie et al., 1993; Foyer et al., 1994; Van Camp et al., 1994b; Slooten et al., 1995). To our knowledge, the present report is the first to describe overproduction of FeSOD in plants. The level of overexpression of FeSOD in line SR1–14, which was studied most extensively, was around 7.4-fold (Fig. 1; plants grown at 135 μmol m⁻² s⁻¹) to 16-fold (Fig. 4E; plants grown at 65 μmol m⁻² s⁻¹). These variations were due mainly to differences in activity of the endogenous FeSOD, depending on the light intensity received during growth (Slooten et al., 1995). After nondenaturing electrophoresis and activity staining, trFeSOD showed up as a double band in transgenic tobacco (Fig. 1, bands 1 and 2). This double band was also observed in extracts from A. thaliana, the source organism of the transgene (not shown). The band splitting is presumably due to a posttranslational modification. In transgenic tobacco, the fast-moving peak was about three times lower in root extracts than in leaf extracts (not shown), suggesting that posttranslational modification occurs to different extents in different organs of the plant. It may be added that the MnSOD also becomes posttranslationally modified when it is overproduced in the chloroplasts (C. Bowler, W. Van Camp, and D. Inzé, unpublished data).

Tolerance to MV

Transgenic plants with different levels of FeSOD overproduction did not differ significantly with respect to enhancement of MV tolerance (Fig. 3). Apparently, the lowest level of SOD overproduction, around 11 units/mg chlorophyll (Table II), was already sufficient to relieve the rate limitation of superoxide scavenging by endogenous chloroplastic SODs.

Judging by the enhanced MV tolerance, SOD-overproducing plants have an enhanced superoxide-scavenging capacity, and therefore they produce more H₂O₂ than control plants during illumination in the presence of MV. This would be highly toxic if it were not removed by ascorbate peroxidase (see the introduction section). However, in the MV experiments, the leaf discs were illuminated with low-intensity light (30 μmol m⁻² s⁻¹). This was done partly to avoid photoinhibition per se, but especially to

Figure 4. Effects of salt stress on FeSOD-overexpressing (SR1–14) and control plants. NaCl was given from d 12 after sowing (see "Materials and Methods"). Data are presented as mean ± se. A, Growth measured as fresh weight accumulation in leaves and stems of transgenic (solid symbols) and control plants (open symbols) (n = 10). B, Ratio of fresh weights between unstressed and salt-stressed plants. C, Dry weights of the leaves and stems of unstressed and salt-stressed plants at 42 d after sowing (n = 5). D, Sodium content of the leaves and stems of transgenic and control plants at 42 d after sowing (n = 5). E, Antioxidant enzyme activities in the leaves of transgenic and control plants at 42 d after sowing (n = 5). From left to right, FeSOD, cytosolic Cu/ZnSOD, chloroplastic Cu/ZnSOD, APx, GR, and DHAR. The activities of GR were multiplied by 10.

Figure 5. APx banding pattern of leaf extracts from FeSOD-overproducing plants (SR1–14) and control plants (SR1). The plants were watered with (solid curves) or without (dashed curves) 60 mM NaCl during growth. Where indicated, the gel was treated with 0.2 mM 4-(chloromercuri)benzenesulphonic acid prior to staining. Migration is from left to right. Band a, Cytosolic APx; bands b and c, chloroplastic APx corresponding with chlAPx-2 and chlAPx-1, respectively.
avoid a situation in which the \( \text{H}_2\text{O}_2 \)-scavenging capacity of endogenous APx would be overwhelmed by a high rate of superoxide production and scavenging. In addition, we have evidence that in tobacco leaf discs, \( \text{H}_2\text{O}_2 \) by itself does not readily inactivate either the PSII reaction center or the plasmalemma (L. Slooten, K. Capiau, S. Kushnir, M. Van Montagu, and D. Inzé, unpublished data). Presumably, both of these factors contributed to the clear-cut protection by trFeSOD of the PSII reaction center against damage induced by superoxide.

We reported previously that in var SR1, overproduction of MnSOD provided protection against MV-induced ion leakage (due to cell membrane deterioration), but not against MV-induced inactivation of PSII (Slooten et al., 1995). In contrast, overproduction of FeSOD resulted in protection against both types of damage. This difference cannot be attributed to differences in activity levels of overproduced SOD, since plants overproducing MnSOD contained on average 13 units/mg chlorophyll of the trFeSOD of the PSII reaction center against damage induced by superoxide.

Overproduction of FeSOD did not enhance the tolerance to photoinductive conditions either (Table V). Mainly from in vitro studies with various PSII preparations, it was concluded that the primary event in photoinhibition is inhibition of whole-chain electron transport, either at the acceptor side or at the donor side of the PSII reaction center (for review, see Prasil et al., 1992; Aro et al., 1993; Barber, 1994). During acceptor-side photoinhibition, active oxygen species formed by the still-functional primary radical pair initiate the degradation of the D1 protein of the reaction center. Most of the attention has been focused on singlet oxygen (Vass et al., 1992; Hideg et al., 1994), but superoxide has been implicated as well (Miyao, 1994). During donor-side photoinhibition, degradation of the D1 protein is not strictly dependent on oxygen, yet the degradation is strongly accelerated by superoxide (Chen et al., 1995), which can be generated by the still-functional primary radical pair. Superoxide production becomes manifest only after complete removal of Mn, which is somehow involved in a SOD-like activity displayed by the reaction center itself (Ananyev et al., 1994; Chen et al., 1995). Donor-side photoinhibition can occur under conditions that destabilize the water-splitting complex, such as exposure to low temperature (Wang et al., 1992). In contrast to acceptor-side photoinhibition, donor-side photoinhibition can occur at low light intensities (Eckert et al., 1991).

In the present study, overproduction of FeSOD did not enhance the tolerance to chilling-induced photoinhibition, either at low or high light intensities (Table V). This is in agreement with the results obtained with MnSOD-overproducing plants (Slooten et al., 1995). There are several possible explanations for this result: (a) photoinhibition was not due to superoxide production in the reaction center; (b) photoinhibition was due to superoxide production in the reaction center, but the superoxide brought about oxidative damage without becoming accessible to overexpressed SOD; and (c) the reaction centers did produce superoxide accessible to transgenic SOD, but this was then converted to \( \text{H}_2\text{O}_2 \), which can be equally damaging (Miyao-Tokutomi et al., 1995). In light of the above discussion, we assume that explanations b and c are more likely than explanation a. In view of the results discussed in the previous section (indicating that trFeSOD does protect the PSII reaction center against superoxide produced by PSII), it would seem that explanation b is more likely than explanation c.

Sen Gupta et al. (1993a, 1993b) found that transgenic tobacco (\( N. \text{tabacum cv Xanthi} \)) overproducing pea Cu/ZnSOD in the chloroplasts exhibited an enhanced tolerance
to inhibition of CO₂ fixation that was induced by high light at low temperatures. This indicates that in the experiments of Sen Gupta et al. (1993a, 1993b), inhibition of CO₂ fixation was caused by the production of superoxide at a rate exceeding the endogenous scavenging capacity. Sen Gupta et al. (1993a, 1993b) used lower temperatures and higher light intensities during the stress treatment than we did. Hence, the rate of superoxide production may have been higher in their experiments than in ours. However, in the experiments of Sen Gupta et al. (1993a), the recovery from chilling-induced inhibition of CO₂ fixation was virtually complete within about 20 min after return to 25°C. Recovery from oxidative damage to the PSII reaction center generally requires several hours (Thiele et al., 1995; cf. Greer et al., 1986). Therefore, it seems unlikely that in the experiments of Sen Gupta et al. (1993a), the inhibition of CO₂ fixation was due to oxidative damage to the PSII reaction center.

Effect of trFeSOD on Induction of Antioxidant Enzymes during Salt Stress

We observed two chloroplastic APx isozymes on APx activity gels (Fig. 5) (L. Slooten, K. Capiau, S. Kushnir, M. Van Montagu, and D. Inzé, unpublished data). These are water-soluble enzymes and are therefore different from the intrinsically membrane-bound APx first found in spinach (Miyake and Asada, 1992). We found a similar membrane-bound APx activity in cuvette assays in membrane preparations from tobacco chloroplasts, but could not visualize this activity on APx activity gels (L. Slooten, K. Capiau, S. Kushnir, M. Van Montagu, and D. Inzé, unpublished data).

In nonstressed tobacco plants, overproduction of FeSOD had no effect on activity levels of other antioxidant enzymes, including APx. This was also the case when the plants were grown at a light intensity of 650 μmol m⁻² s⁻¹ (see “Materials and Methods”) (not shown). This agrees with earlier data obtained with transgenic plants overproducing MnSOD in the chloroplasts (Slooten et al., 1995). However, when we applied salt stress, only the transgenic, FeSOD-overproducing plants exhibited at least a 3-fold increase in activity of chlAPx-1. In contrast, chlAPx-2 was enhanced in both the transgenic and the nontransgenic plants in response to salt stress (Fig. 5). This suggests that at least chlAPx-1 is induced by H₂O₂. During salt stress, H₂O₂ is probably generated at higher rates in transgenic, FeSOD-overproducing plants than in nontransgenic plants. The differential response of the two chloroplastic APx isozymes to salt stress indicates some functional differentiation of these isozymes. In var SRI, chlAPx-1 represents only a minor fraction of the total chloroplastic APx. We noted that leaf extracts from cv PBD6 contained higher activities of chlAPx-1 than leaf extracts from cv SRI after electrophoresis on nondenaturing gels (L. Slooten, K. Capiau, S. Kushnir, M. Van Montagu, and D. Inzé, unpublished data). Sen Gupta et al. (1993b) reported that transgenic tobacco (N. tabacum cv Xanthi) overproducing pea Cu/ZnSOD in the chloroplasts exhibited a 3-fold increase in APx activity. The difference between the results of their experiments and ours may be due to differences in the APx isozyme distribution pattern between cv Xanthi and cv SRI.

In accordance with data from other groups, we found that salt stress leads to increases in the activity of many antioxidant enzymes (chloroplastic FeSOD, cytosolic Cu/ZnSOD, chloroplastic Cu/ZnSOD, APx, GR, and DHAR) in nontransgenic plants, indicating that oxidative stress is one of the components of salt stress (Fig. 4E). In the absence of stress, the activities of all of these enzymes except FeSOD were the same in nontransgenic and FeSOD-overproducing plants. However, the increase in activity of cytosolic and chloroplastic Cu/ZnSOD, observed in control plants under salt stress, was suppressed in FeSOD-overproducing plants (Fig. 4E). Cytosolic Cu/ZnSOD and trFeSOD are located in different cellular compartments, indicating that trFeSOD interferes with a signal pathway leading to induction of cytosolic Cu/ZnSOD, and probably also of chloroplastic Cu/ZnSOD. This interference is probably due to the fact that trFeSOD lowers the concentration of superoxide in the chloroplasts. Similar results were obtained previously with plants overproducing a mitochondrial MnSOD in the chloroplasts (Slooten et al., 1995). It remains to be determined whether superoxide itself or a molecule derived from a reaction with superoxide constitutes the signal for induction of cytosolic Cu/ZnSOD.

Salt stress is complex, imposing a water deficit because of osmotic effects and exerting ion-specific toxic effects on a wide variety of metabolic activities (Greenway and Munns, 1980; Cheeseman, 1988). The water deficit might be expected to cause oxidative stress, which is similar to drought stress (Bowler et al., 1992). Indeed, Singh and Choudhuri (1990) reported that superoxide and H₂O₂ could be important in the mechanism of salt injury. This notion was corroborated by subsequent reports indicating an increase in the activities of antioxidant enzymes in response to high salinity, and by correlations of salt tolerance with antioxidant enzyme levels (Gossett et al., 1994; Olmos et al., 1994; Hernandez et al., 1995; Sehmer et al., 1995). In our experiments the chloroplasts of salt-stressed transgenic plants exhibited elevated activities not only of SOD but also of APx in comparison with salt-stressed control plants (see above). Nevertheless, FeSOD-overproducing plants were no more tolerant to salt stress (as indicated by biomass accumulation; Fig. 4, A–C) than were control plants. This suggests that in the present experiments, growth of salt-stressed plants was not limited by the capacity of the chloroplasts to scavenge superoxide and H₂O₂. It cannot be excluded that the lack of induction of cytosolic Cu/ZnSOD in transgenic plants overproducing FeSOD contributed to the lack of enhancement of salt tolerance in these plants.

CONCLUSIONS AND PROSPECTS

The results obtained with transgenic plants overproducing either FeSOD or MnSOD in the chloroplasts indicate that FeSOD provides better protection against MV-dependent oxidative stress than does MnSOD. We attribute this tentatively to the higher membrane affinity of trFeSOD, allowing this enzyme to scavenge superoxide radicals at the site of their formation, i.e. near PSI. This difference between Mn-
SOD and FeSOD is probably connected to the original subcellular localization of these enzymes. From all this it may be anticipated that FeSOD-overproducing plants will be more tolerant to physiological stresses entailing enhancement of light-induced superoxide formation than MnSOD-overproducing plants. FeSOD-overproducing plants were not more tolerant to salt stress than control plants, indicating that, at least under the present assay conditions, the superoxide-scavenging capacity was not a limiting factor for growth under salt stress. However in salt-stressed plants the overproduced enzyme interfered with signal pathways for induction of antioxidant enzymes in such a manner that induction of one chloroplastic APx isozyme was promoted and induction of cytosolic and chloroplastic Cu/ZnSOD was inhibited. Thus, these plants may provide clues for the elucidation of signal pathways involved in induction of other antioxidant enzymes. Furthermore, it seems likely that FeSOD-overproducing plants can provide interesting material to assess the importance of oxidative stress in various physiological types of stress, and in addition, provide a good starting point for studying cooperative effects between different overproduced antioxidant enzymes in the scavenging of toxic oxygen species.

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