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Overproduction of *Arabidopsis thaliana* FeSOD Confers Oxidative Stress Tolerance to Transgenic Maize

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Transgenic maize (*Zea mays* L.) plants have been generated by particle gun bombardment that overproduce an *Arabidopsis thaliana* iron superoxide dismutase (FeSOD). To target this enzyme into chloroplasts, the mature *Fesod* coding sequence was fused to a chloroplast transit peptide from a pea ribulose-1,5-bisphosphate carboxylase gene. Expression of the chimeric gene was driven by the CaMV 35S promoter. Growth characteristics and in vitro oxidative stress tolerance of transgenic lines grown in control and chilling temperatures were evaluated. The transgenic line with the highest transgenic FeSOD activities had enhanced tolerance toward methyl viologen and had increased growth rates.

Key words: Chilling — Methyl viologen — Oxidative stress — Superoxide dismutase (EC 1.15.1.1) — Transgenic plant — *Zea mays*.

In chilling-sensitive plants, oxidative stress is a major component of chilling stress (Hodges et al. 1997, Pinhero et al. 1997). Chilling temperatures in combination with high light intensities provoke an enhanced production of activated oxygen species (AOS), such as superoxide ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2) (Wise and Naylor 1987). Oxygen radicals can react very rapidly with DNA, lipids, and proteins, which causes severe cellular damage. Under normal conditions, AOS are efficiently scavenged by both enzymatic and non-enzymatic detoxification mechanisms. During prolonged stress conditions, however, such detoxification systems get saturated and damage occurs. The main players within the defence system are superoxide dismutases (SODs), ascorbate peroxidases (APXs) and catalases (Inzé and Van Montagu 1995). SODs, which can be considered as key enzymes within the antioxidative stress defense mechanism, directly determine the cellular concentration of $O_2^{\cdot -}$ and H_2O_2, because they dismutate superoxide into O_2 and H_2O_2. Besides a few exceptions, SODs are present in all aerobic organisms and in all subcellular compartments that have to deal with activated oxygen species. They are classified according their metal co-factor as copper/zinc (Cu/Zn), manganese (Mn), and iron (Fe) SODs. The subcellular distribution of these isozymes is also distinctive (Bowler et al. 1994). The MnSOD is found in the mitochondria of all eukaryotic cells, Cu/ZnSODs in both cytosol and chloroplasts of higher plants, and FeSODs only in prokaryotes and in plant chloroplasts. The amount and relative abundance of the SOD isozymes varies within each organism. Developmental control and environmental stresses that generate the production of AOS (such as UV, ozone, air pollutants, low temperatures, salt stress, drought, heat shock, and pathogen infections) are shown to induce plant SODs (Van Camp et al. 1994a).

Different SODs have been genetically engineered into plants to assess their potential capacities for enhancing oxidative stress tolerance (Bowler et al. 1991, McKersie et al. 1993, Slooten et al. 1995). The beneficial effects observed in some of these transgenic plants could lead to interesting agronomic applications. Overproduction of *Nicotiana plumbaginifolia* MnSOD in alfalfa confers resistance to freezing stress, whereas tobacco plants overproducing the same construct confers visible ozone tolerance (Van Camp et al. 1994b, McKersie et al. 1996). During in vitro generated oxidative stress (Van Camp et al. 1996), plasma membranes and PSII were better protected in transgenic tobacco plants overproducing *Arabidopsis thaliana* FeSOD. To test the effect of SOD in maize, we produced transgenic maize lines overproducing *Arabidopsis thaliana* FeSOD and assessed the performance of these plants during oxidative stress.

Abbreviations: AOS, active oxygen species; APX, ascorbate peroxidase; *Fesod*, gene encoding iron SOD; MV, methyl viologen; PPT, phosphinothricin; SOD, superoxide dismutase.

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Materials and methods

Plasmids—Plasmids used for maize transformation were pUC19-derived vectors. pKA-4 contains the coding sequence of the *Fesod* gene from *Arabidopsis thaliana* (Van Camp et al.

1990) under transcriptional control of the 35S promoter of the cauliflower mosaic virus (CaMV) (Odell et al. 1985) and sequences derived from the 5′ untranslated region of the *SS3.6* gene. The *SS3.6* gene encodes a small subunit of ribulose 1,5-bisphosphate carboxylase from *Pisum sativum*. The *Fesod*-coding region was translationally fused to the chloroplast transit peptide from the pea ribulose-1,5-bisphosphate carboxylase (*SS3.6* gene) (Cashmore et al. 1983). The 3′ untranslated region was derived from the T-DNA gene *7* (Velten and Schell 1985). pDE110 contains the coding region of phosphinothricin acyltransferase from *Streptomyces hygroscopicus* (*bar* gene) conferring resistance against phosphinothricin (Thompson et al. 1987) under control of the CaMV 35S promoter. The 3′ untranslated region is derived from the nopaline synthase gene (3′nos) (Depicker et al. 1982).

Production of transgenic maize lines overproducing Arabidopsis thaliana FeSOD—Transgenic maize (*Zea mays* L.) plants were generated by particle bombardment of type 1 callus derived from immature zygotic embryos from a hybrid between inbred lines H99 and Pa91 [(Pa91 × H99) × H99]. pKA-4 and pDE110 plasmids were linearized with *Xba*I and *Hin*dIII, respectively. Expression of the *bar* gene in regenerated R_0 plants was monitored by a phosphinothricin treatment in a paint assay in the greenhouse (De Block et al. 1987). Ninety-four independent lines were produced. The presence and expression of the cotransformed *Fesod* gene construct was tested by PCR analysis. Ninety percent of the lines were cotransformed with the pKA-4 vector, of which 71 lines were tested for *Fesod* expression by RNA blot analysis. Four lines had detectable *A. thaliana Fesod* transcript levels. Primary transformants were cross-pollinated with a wild-type plant (H99). Segregation analysis was done by spraying 20 R_1 seedlings of each back-cross with phosphinothricin.

SOD activity gel assay—Soluble proteins were extracted from maize leaves and assayed for SOD activity on native 10% polyacrylamide gels using the in situ staining technique of Beauchamp and Fridovich (1971), as previously described by Van Camp et al. (1990). Incubation of the gels with KCN inactivated Cu/ZnSOD isoforms; incubation with H_2O_2 inhibited FeSOD and Cu/ZnSOD isoforms.

DNA gel blot analysis—Genomic DNA was extracted as described by Dellaporta et al. (1983). Genomic DNA was digested with the appropriate restriction enzymes, run on a 1% agarose gel, transferred to nylon Hybond-N+ membranes (Amersham, Aylesbury, U.K.), and hybridized with ^{32}P-radioactive probes that were labelled with Megaprime (Amersham) or T7 Quick Prime (Pharmacia, Uppsala, Sweden).

Growth conditions in stress assessments—Seeds were sterilized with 0.2% $HgCl_2$ for 2 min, rinsed with distilled water, and sown on moistened vermiculite. After 5 d of germination in the dark at room temperature, seedlings with a root of 5–7 cm and a hypocotyl of 1–2 cm were transferred to pots containing a bottom layer of 3 cm vermiculite and a 6-cm thick layer of soil. The soil consisted of 45% white peat, 45% black peat, and 5% sand, and was enriched with fertilizer containing trace elements. The plantlets were kept for 4 d at room temperature, shielded from direct sunlight, and were then transferred (at day 0) to the growth chamber (SANYO Gallenkamp, Loughborough, U.K.). From that day on, the plants were watered with a full-strength Hoagland solution (Hoagland and Arnon 1938), except that ammonium phosphate was replaced by 1 mM K_2HPO_4. In the growth chamber, the plants were grown in a 16-h light/8-h dark cycle at approximately 90% relative humidity, and at day/night temperatures of either 17/15°C or 25/22°C. The light intensity was 1.6 μmol m^{-2} s^{-1} during the first 0.5 h ("dawn"), 900 μmol m^{-2} s^{-1} during the next 15 h ("day"), and again 1.6 μmol m^{-2} s^{-1} during the last 0.5 h ("dusk"). The dawn and dusk light was provided by incandescent lamps, and the daylight by fluorescent tubes. The pots were shifted around every day in the growth chamber to avoid adaptation of the plants to local differences in temperature or light intensity.

Methyl viologen tolerance leaf disc assays—Leaf discs from the third leaf were incubated overnight in the dark at room temperature with water or an aqueous solution of methyl viologen (MV). The discs were then illuminated for 2 h at 30 μmol m^{-2} s^{-1} provided by cool-white fluorescent tubes, and were subsequently dark-incubated for another 20 h at 28°C. The MV-dependent oxygen radical damage was estimated firstly from ion leakage out of the leaf discs due to destruction of membrane lipids. Ion leakage was measured as an increase in the conductance of the floating solution. As a measure of the MV-dependent decrease in activity of the reaction center of PSII, the variable chlorophyll fluorescence ratio F_v/F_{max} was used. This ratio gave the exciton trapping efficiency when all photochemical traps were open (Genty et al. 1989). The fluorescence measurements were made with a PAM fluorometer (Walz, Effeltrich, Germany). The average values of three to four leaf discs at each MV concentration (including zero) were used to calculate the MV-induced increase in conductance of the floating solution or the MV-induced decrease in F_v/F_{max}.

Results

Transgenic maize lines with enhanced FeSOD activity—Presence of transgenic FeSOD in chloroplasts enhances the oxidative stress tolerance in transgenic tobacco plants (Van Camp et al. 1996). To test the protective effect of FeSOD in maize, we made a chimeric gene construct in which the *A. thaliana Fesod*-coding region was fused in frame with the chloroplast transit peptide of a small subunit of a pea ribulose-1,5-bisphosphate carboxylase (Cashmore et al. 1983). This chloroplast transit peptide (tp) directed effectively recombinant proteins in maize chloroplasts (Van Breusegem et al. 1998). The cassette was under transcriptional control of the CaMV 35S promoter (Odell et al. 1985). Maize type 1 callus was transformed with plasmids pKA-4 and pDE110 that contained CaMV35S-tp-*Fesod* and CaMV35S-*bar*, respectively (Fig. 1; Materials and Methods).

One hundred and ten plants were regenerated from phosphinothricin (PPT)-resistant calli and transferred to the greenhouse. These plants were subsequently rescreened for expression of the *bar* gene by a herbicide treatment in the greenhouse. Ninety-four R_0 PPT-resistant transgenic lines were tested for the presence of the *Fesod* gene construct by PCR. As 82 plants scored positively for the presence of the transgene, 90% frequency of cotransformation was found. Seventy-one plants were tested for *Fesod* expression by RNA gel blot analysis. Transgenic transcript could only be found in four plants originating from independent transformation events. These four lines (denominated FES1 to FES4) were back-crossed with line H99 and produced seeds.

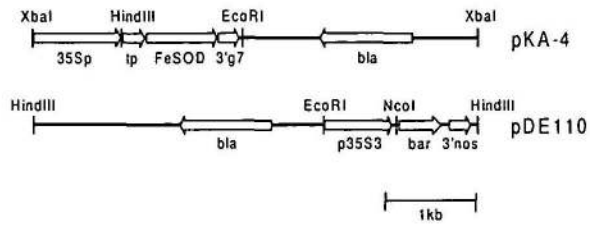

Fig. 1 Schematic presentation of the constructs used for overexpression of *Arabidopsis thaliana Fesod* in transgenic maize. Plasmid pKA-4 contains the *Arabidopsis thaliana Fesod* cDNA fused to the N-terminal chloroplast transit peptide (tp) from a pea small subunit of ribulose-1,5-bisphosphate carboxylase (Van Camp et al. 1996). Plasmid pDE110 contains the *bar* gene as a selectable marker. Both cassettes are flanked at the 5′ end by a CaMV 35S promoter (35Sp; p35S3) and at the 3′ end by polyadenylation signals of the T-DNA gene 7 (3′g 7) or the nopaline synthase gene (3′nos). The *bla* gene codes for β-lactamase, which provides ampicillin resistance.

All four back-crossed lines were assayed for extra transgenic FeSOD activity. Figure 2 shows the SOD banding pattern on a non-denaturing gel of leaf extracts from a wild-type and a transgenic plant from lines FES1 and FES2. The gels were stained in the absence or presence of KCN. In leaf extracts of non-transgenic plants, four bands (MnSOD and Cu/ZnSOD) were observed. In FES2 leaf extracts, a clear extra SOD activity band was present. This SOD activity was resistant to KCN and sensitive to H_2O_2 and is, therefore, an FeSOD isoform. In FES1, lower amounts, almost undetectable, of transgenic FeSOD were present. Unfortunately, in the line with the highest transgene expression levels (FES4), the transgene was not inherited. In FES3, no FeSOD activity could be detected, despite the presence of the transgene. The two transgenic lines (FES1 and FES2), which had detectable levels of transgenic FeSOD activity, were used for further evaluation.

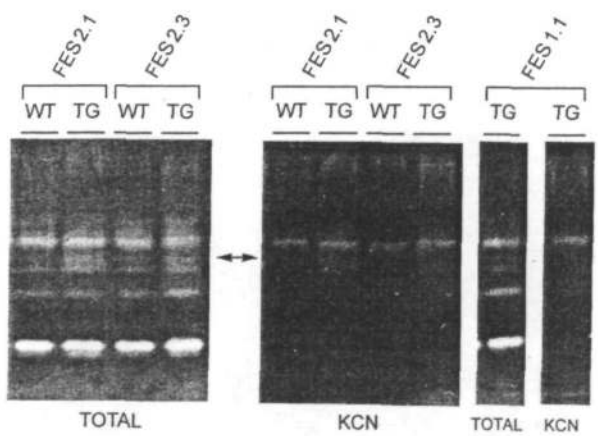

Fig. 2 Characteristics of *Arabidopsis thaliana* FeSOD in leaves of transgenic maize. SOD isozyme patterns of protein extracts from wild-type and transgenic maize plants overproducing *Arabidopsis thaliana* FeSOD. The non-denaturing polyacrylamide gel was loaded with 50 μg protein per slot and negatively stained with riboflavine-nitroblue tetrazolium. Samples were taken of leaf 4 after 26 d of growth at 17/15°C of a transgenic (TG) and wild-type plant (WT), originating from lines FES1 and FES2. An arrow indicates the transgenic FeSOD activity.

Transgenic R_1 plants were back-crossed with the parental line H99 to obtain R_2 seed stocks. Figure 3 presents a DNA gel blot analysis of R_2 plants (H99 × (FES × H99)) of FES1 and FES2, digested with *Hin*dIII/*Eco*RI, which releases the *Fesod*-coding region plus the 3′ g7 region, and hybridized with a coding region fragment from the *Arabidopsis thaliana Fesod* cDNA. FES1 and FES2 R_2 plants have simple integration patterns: FES1 had one hybridizing 1.2-kb band that corresponded to the intact *Fesod* fragment. FES2 had two larger weakly hybridizing bands. When hybridizing the same filters with a *bar* probe, again a simple integration pattern was observed in the two lines. By using other restriction digests and gene-specific probes (data not shown) we could conclude that FES2 R_2 progeny plants contained two copies of both the *Fesod* and *bar* gene. One set of gene constructs was intact and the other was probably truncated and/or rearranged. FES1 R_1 progeny plants had one intact copy of both the

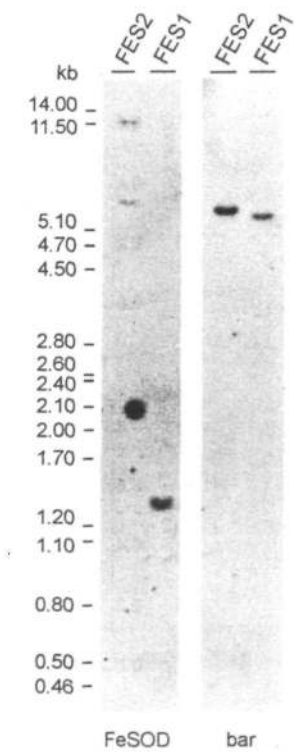

Fig. 3 Integration patterns of the *Fesod* and *bar* gene constructs in transgenic maize lines. Genomic DNA of R_2 transgenic maize lines was digested with *Hin*dIII/*Eco*RI and hybridized with a 994-bp ^{32}P-labelled *Hin*dIII-*Bam*HI fragment of the *Fesod* cDNA or a 546-bp ^{32}P-labelled *Nco*I-*Bgl*II *bar* fragment.

bar and *Fesod* gene and one truncated copy of the *bar* gene.

Growth characteristics and oxidative stress tolerance of transgenic and wild-type plants—Different R_2 seed lots from line FES1 [H99 × (FES1 × H99)] and line FES2 [(FES2 × H99) × H99] were generated by back-crossing transgenic R_1 plants with the parental line H99. Consequently, in each seed lot, half of the plants were heterozygous for the transgenes, whereas the other half did not possess the transgenes. These segregating populations were used in each of the following experiments with the wild-type plants as negative internal controls. Approximately, 20 wild-type and 20 transgenic plants were used in each experiment. Plants of the two lines were grown at day/night temperatures of either 25/22°C or 17/15°C. Plant growth characteristics were followed and oxidative stress tolerance was assessed with an in vitro leaf disc MV assay (see Materials and Methods). Because most of the damage is light dependent (Slooten et al. 1995; data not shown), the oxidative damage caused by MV, which is a PSI electron acceptor, is mainly found in the chloroplasts, presumably in the vicinity of PSI.

Leaf discs of transgenic plants have an increased MV tolerance—Figure 4A presents the MV tolerance of transgenic plants from line FES2 (seed lot FES2.3) when grown at 25/22°C. Leaf discs from the third leaf of transgenic plants suffered less inactivation of PSII and less ion leakage than those from wild-type plants, as indicated by F_v/F_{max} and conductivity measurements, respectively. These results indicate that transgenic plants were slightly more tolerant to MV than wild-type plants. Similar results were obtained when plants were continuously grown at 17/15°C (Fig. 4B).

MV tolerance measurements were done with one seed lot from FES1 and with three independent seed lots from FES2. The results are summarized in Figure 5. Plants grown at 25/22°C were more tolerant to MV than plants grown at 17/15°C. To compensate for this effect, the results obtained with 0.9 μM MV in plants grown at 25/22°C (Fig. 5A, B) were compared with data obtained at 0.6 μM MV in plants grown at 17/15°C (Fig. 5C, D). After growth at 25/22°C, transgenic plants showed on average an enhanced MV tolerance, as compared with wild-type plants, in all seed lots of FES2 (Fig. 5A, B). This was observed in the conductivity measurements (Fig. 5A), as well as in the fluorescence measurements (Fig. 5B). At 17/15°C, the results were less consistent in this respect

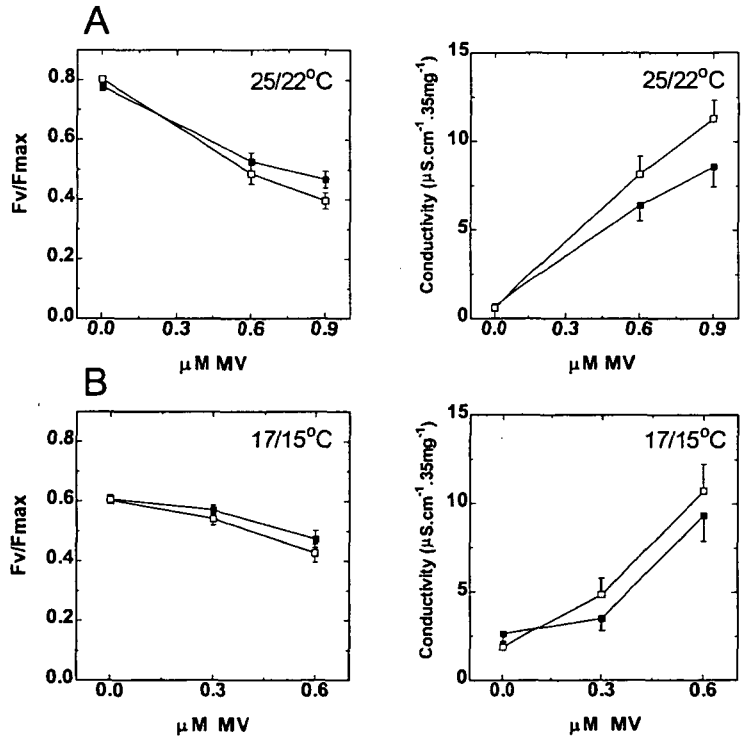

Fig. 4 MV tolerance of FES2. Plants from seed lot FES2.3 were grown at 25/22°C or 17/15°C. Mean ± SE. Left panels: F_v/F_{max} in leaf discs from the third leaf, after treatment of leaf discs with the indicated concentration of MV (three leaf discs per plant were used for each MV concentration). Right panels: conductivity of the floating solution after treatment of leaf discs with the indicated concentration of MV. Conductivity is expressed in $\mu S\ cm^{-1}$ (35 mg FW)$^{-1}$. (A) Plants grown at 25/22°C. n = 22 for wild-type plants (open symbols), n = 23 for transgenic plants (solid symbols). (B) Plants grown at 17/15°C. n = 20 for wild-type plants, n = 19 for transgenic plants.

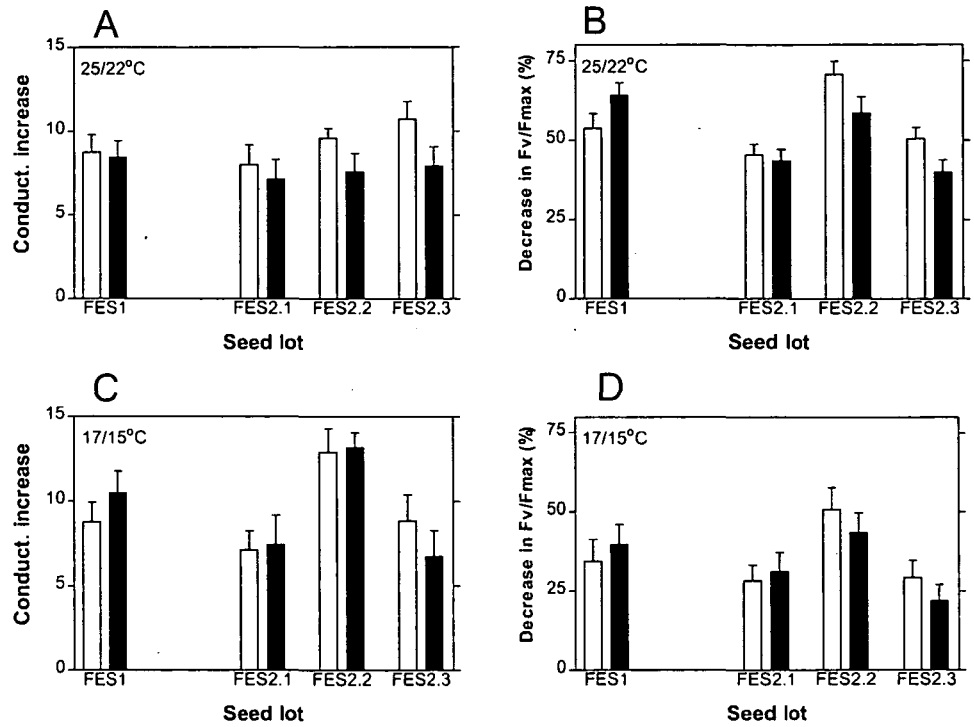

Fig. 5 MV effects on wild-type (open bars) and transgenic plants (black bars) of two different FeSOD-producing lines (FES1 and FES2) after growth at 25/22°C (panels A and B) or 17/15°C (panels C and D). Mean±SE. A, C, MV-induced increase in the conductivity of the floating solution (in $\mu S\ cm^{-1}$ (35 mg FW)$^{-1}$). B, D, MV-induced decrease in F_v/F_{max}, in percent of the F_v/F_{max} measured without MV. Results were obtained with 0.9 μM MV (A, B) and 0.6 μM MV (C, D).

(Fig. 5C, D): Only the second and third seed lot of FES2 showed an enhanced MV tolerance of transgenic plants compared to that of controls, and in the FES2.2 this was the case only in measurements of PSII activity (Fig. 5D). In line FES1, enhancement of MV tolerance was not observed (data not shown). This observation correlates with the lower levels of transgenic FeSOD activity in line FES1 (Fig. 2).

Transgenic plants have better growth characteristics—Figures 6 and 7 show the growth characteristics of line FES2 grown at 25/22°C and 17/15°C. The transgenic plants grew somewhat faster than wild-type plants as estimated from the rate of leaf elongation (Fig. 6, 7A), the rate of increase in total leaf length (Fig. 7B), the fresh weight of the leaves at the time of harvest (Fig. 7C), and the fresh weight of the whole plant at the time of harvest (Fig. 7D).

Growth measurements were done with all available seed lots of the two lines at both control and chilling temperatures. These growth data are summarized in Figure 8. Transgenic plants from line FES2 grew slightly faster than wild-type plants at control and chilling temperatures. In this respect, the data on the summed leaf length at the time of harvest and the fresh weight of the plants are in good agreement. In line FES1, the growth rate between transgenic and wild-type plants was essentially the same.

FeSOD overproduction does not specifically enhance chilling tolerance—The weight ratio (i.e., the ratio of the weight of the plants grown at 17/15°C over that at 25/22°C) from the two lines is shown in Figure 9. In line FES1, a seed lot (FES1.2) that contained no transgenics was included into the experiment. There was no significant difference or tendency between transgenic and wild-type plants with respect to the weight ratio. This suggests that overexpression of *Fesod* does not specifically enhance chilling tolerance, but provides the transgenic plants with generally improved growth characteristics.

Differences between transgenic and wild-type plants are highly significant in FES2—On average, transgenic plants of line FES2 grew faster than wild-type plants in all experiments; in this respect, there was no consistent difference between the three seed lots (Fig. 8). Therefore, in each growth experiment performed with FES2 we calculated the mean and SEM of the difference between transgenic and wild-type plants as well as the significance of the difference, P, in a one sided *t* test. At either growth temperature, we considered the experiments performed with the three seed lots of FES2 as a series of three identical repeats. Thus, we could use the statistic $\chi^2 = 2\Sigma u_i$, where $u_i = -\ln P_i$, with P defined as above and the index i running from 1 to n, with n the number of repeats. If there is no

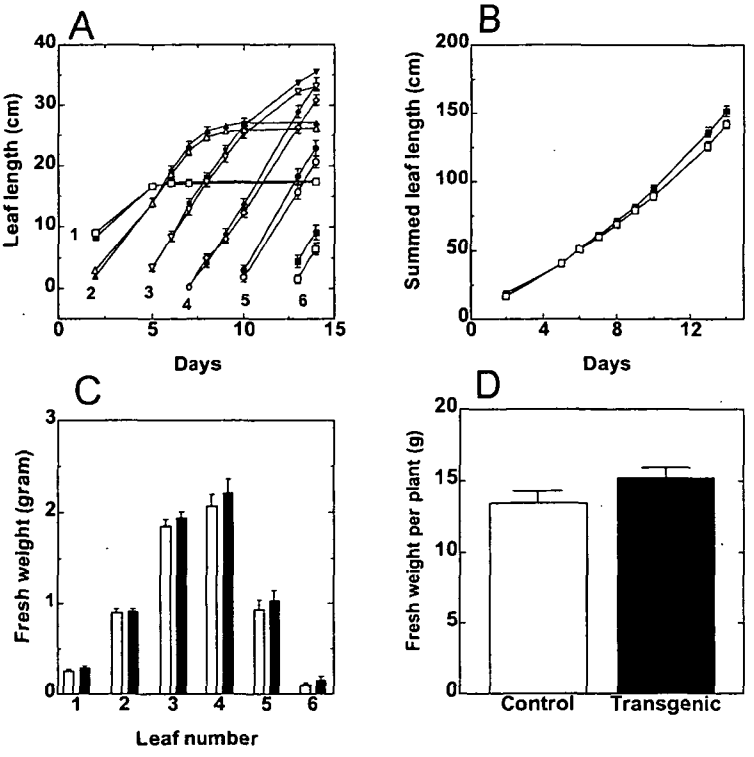

Fig. 6 Growth characteristics of FES2. Plants from seed lot FES2.3 were grown at 25/22°C. n=22 for wild-type plants (open symbols, open bars), n=23 for transgenic plants (closed symbols, black bars). Mean±SE. (A) Leaf elongation. Leaf numbers are indicated. (B) Summed leaf length. (C) Fresh weight of the leaves at the time of harvest. (D) Fresh weight of the whole plant at the time of harvest.

significant difference between transgenic and wild-type plants (i.e., when the average P value is 0.5), then the sampling distribution of the statistics is approximated by the χ^2 distribution with 2n degrees of freedom (Winer 1970). This approach allows one to calculate an overall significance level for the observed differences. The results are shown in Table 1.

Overall, transgenic plants derived from FES2 exhibited better growth than control plants at 25/22°C; the difference in growth characteristics (fresh weight and summed leaf length at the time of harvest) was highly significant. In addition, after growth at 25/22°C, transgenic plants were overall significantly more tolerant to MV-induced oxidative stress than wild-type plants. The protection was exerted at the level of PSII activity (as evidenced from F_v/F_{max}) and at the plasma membrane (reflected in the ion leakage). By and large, similar tendencies were observed after growth at 17/15°C, but the differences between transgenic and wild-type plants were not significant, either in individual seed lots or overall.

In line FES1, no significant differences between transgenic and wild-type plants was observed in any of these characteristics irrespective of the growth regime (data not shown).

Table 1 Significance of the differences between transgenic and wild-type plants in FES2

Parameter	Significance	
	25/22°C	17/15°C
Fresh weight[a]	>0.995	NS
Summed leaf length[b]	>0.995	>0.90
F_v/F_{max}[c]	>0.98	NS
Conductivity[c]	>0.97	NS

[a] Of whole plants at the time of harvest.
[b] Summed leaf length at the time of harvest.
[c] In the presence of 0.9 or 0.6 μM MV after growth at 25/22°C or 17/15°C, respectively.
NS, not significant.

Discussion

A wide variety of environmental stresses cause the production of AOS within plants. Tolerance toward oxidative stress has been correlated with enhanced levels of SODs in plants. Transgenic plants overproducing SODs show in most cases enhanced tolerance toward the experienced stresses (Bowler et al. 1991, Sen Gupta et al.

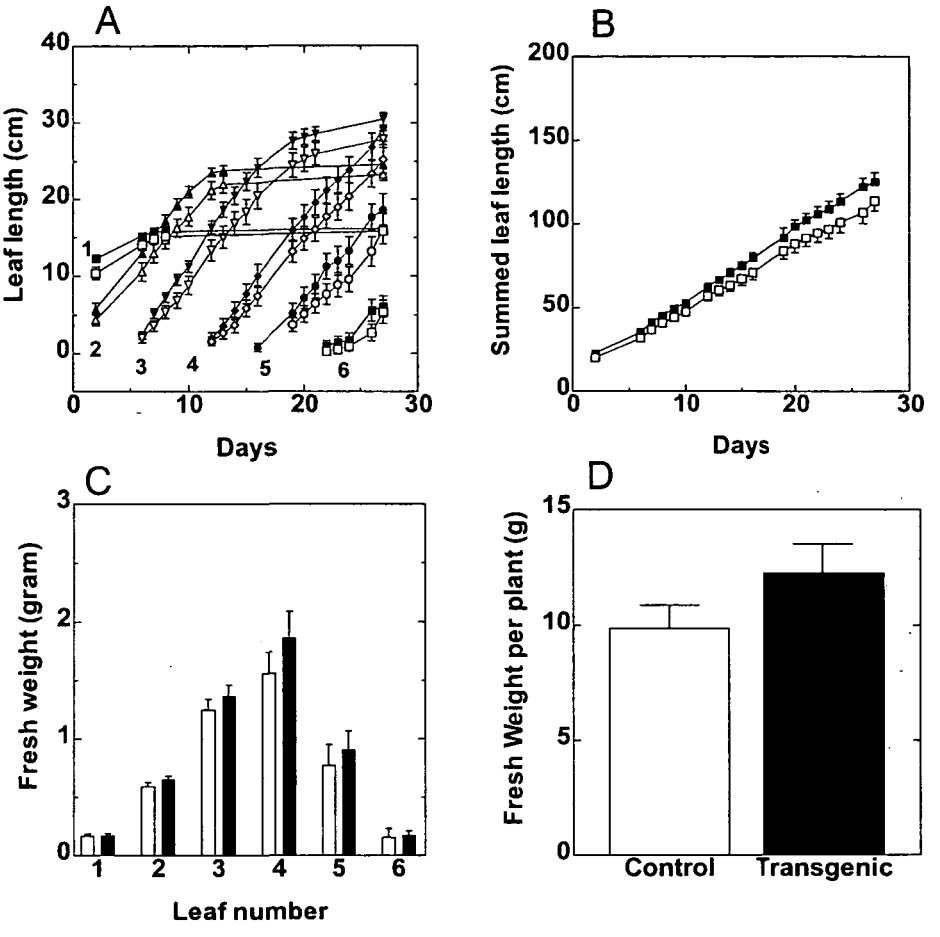

Fig. 7 Growth characteristics of FES2. Plants from seed lot FES2.3 were grown at 17/15°C. n=20 for wild-type plants (open symbols, open bars), n=19 for transgenic plants (closed symbols, black bars). Mean±SE. (A) Leaf elongation. Leaf numbers are indicated. (B) Summed leaf length. (C) Fresh weight of the leaves at the time of harvest. (D) Fresh weight of the whole plant at the time of harvest.

1993a, b, McKersie et al. 1993, 1996, Slooten et al. 1995, Van Camp et al. 1996).

To enhance oxidative stress tolerance in maize, we have generated transgenic maize lines that overproduce *Nicotiana plumbaginifolia* MnSOD (Van Breusegem et al. 1999) and *Arabidopsis thaliana* FeSOD in the chloroplast. By particle bombardment, transgenic maize plants were generated. Despite the production of 82 independent primary transgenic maize plants containing both the *Fesod* and *bar* transgenes, only two lines exhibited enhanced recombinant FeSOD activities. The same observation was made when transforming maize with a similar vector containing *N. plumbaginifolia Mnsod* (Van Breusegem et al. 1999). Extensive rearrangements of the gene constructs during particle bombardment or cosuppression effects could be responsible for the low amounts of primary transformants highly overexpressing the transgene.

The two lines have different expression levels of the *A. thaliana Fesod* transgene, both at the mRNA and activity level. In agreement with the FeSOD activity levels, increased growth rates or enhanced MV tolerance were observed only in one line (FES2). Probably the engineered FeSOD activity is not high enough in line FES1 to have a pronounced beneficial effect on the growth or MV resistance of the plants. Presumably the added activity does not exceed the natural variation between the different plants.

The use of segregating populations of transgenic and control plants enabled us to calculate, within each seed lot, the difference between transgenic and control plants for any given characteristic. In this way, we reduced the influence of the variability, which occurs from one seed lot to another, on the significance of the difference between transgenic and control plants. The transgenic plants in line FES2 exhibited a significantly faster growth than wild-type plants (as estimated from fresh weight and summed leaf length determinations) and also a significantly enhanced MV tolerance (Table 1).

In plants grown at 17/15°C, similar tendencies were

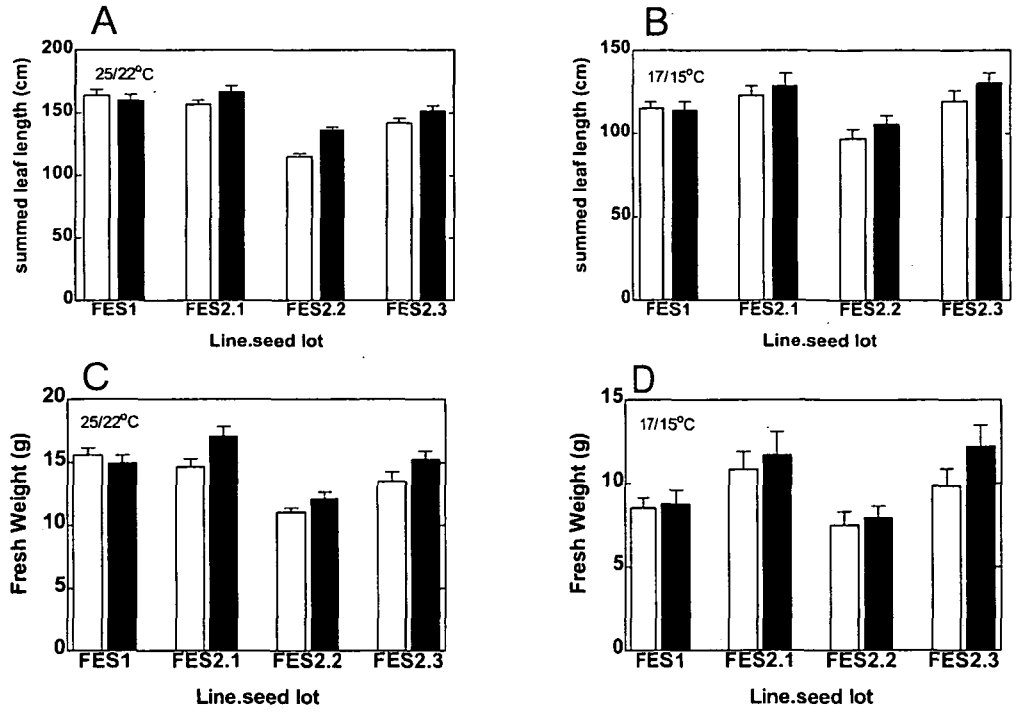

Fig. 8 Summed leaf length (A and B) and plant fresh weight at the time of harvest (C and D) after growth at 25/22°C (A, C) or 17/15°C (B, D). Mean±SE. Open bars, wild-type plants; black bars, transgenic plants. In each case n=±20. Seed lot numbers are indicated in the abscissa.

observed, but the differences were not statistically significant either in individual seed lots or overall. With regard to the growth data, the lack of significance was mainly due to the fact that the errors on the examined characteristics were larger in plants grown at 17/15°C than at 25/22°C (Fig. 8); in other words, the added cold stress increased the variability during growth at 17/15°C. Similar trends were observed with respect to the MV tolerance data.

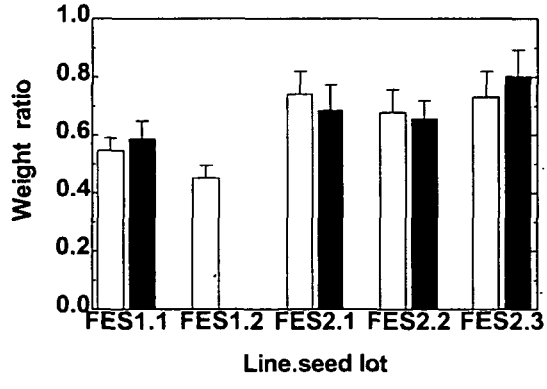

Fig. 9 Weight ratio (as defined in the text) of wild-type (open bars) and transgenic plants (black bars) of two different *Fesod*-expressing lines (FES1 and FES2).

There are some interesting differences between the effects of overexpression of *Fesod* and *Mnsod* in plants. Firstly, in tobacco, overproduced MnSOD protects the plasmalemma, but not the PSII reaction center against MV-induced oxidative damage (Slooten et al. 1995). FeSOD overproduction protects both the plasmalemma and the PSII reaction center against MV. These results, based on conductivity and F_v/F_{max} measurements, were discussed in connection with a postulated difference in suborganellar location between the overproduced MnSOD and FeSOD. Because of their chloroplastic and mitochondrial origin, respectively, FeSOD and MnSOD might have different properties. Van Camp et al. (1996) showed that in tobacco the transgenic FeSOD is at least partially bound to the chloroplast membrane, whereas transgenic MnSOD behaves like a stromal enzyme. This differential subcellular location might provoke different protective effects against oxygen radicals. The major site of MV-induced oxygen radical formation is located within the chloroplast membrane in the Fe-S center B in PSI (Fujii et al. 1990), from which the oxygen radicals may diffuse along or within the membrane to the PSII reaction center (resulting in a decrease of F_v/F_{max}) or alternatively through the stroma to the cell membrane (resulting in an increased ion leakage). Transgenic FeSOD may be able to bind electrostatically to the membrane in the vicinity of the site of radical produc-

tion. In this way it would prevent the spreading of the radicals, and hence it would protect both PSII and the cell membrane against damage. Stromal MnSOD would not be able to scavenge the radicals that are diffusing through the stroma and, hence, only show a protection against ion leakage (Van Camp et al. 1996). The data obtained in transgenic maize with overproduced *Mnsod* (Van Breusegem et al. 1999) and FeSOD are very similar to those obtained in tobacco with the same *Mnsod* and *Fesod* gene constructs, respectively.

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References

Beauchamp, C.O. and Fridovich, I. (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* 44: 276–287.

Bowler, C., Slooten, L., Vandenbranden, S., De Rycke, R., Botterman, J., Sybesma, C., Van Montagu, M. and Inzé, D. (1991) Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. *EMBO J.* 10: 1723–1732.

Bowler, C., Van Camp, W., Van Montagu, M. and Inzé, D. (1994) Superoxide dismutase in plants. *CRC Crit. Rev. Plant Sci.* 13: 199–218.

Cashmore, A.R. (1983) Nuclear genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. *In* Genetic Engineering of Plants—an Agricultural Perspective (Basic Life Sciences, Vol. 26). Edited by Kosuge, T., Meredith, C.P. and Hollaender, A. pp. 29–38. Plenum Press, New York.

De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gosselé, V., Movva, R., Thompson, C., Van Montagu, M. and Leemans, J. (1987) Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *EMBO J.* 6: 2513–2518.

Dellaporta, S.L., Wood, J. and Hicks, J.B. (1983) A plant DNA minipreparation: version II. *Plant Mol. Biol. Rep.* 1: 19–21.

Depicker, A., Stachel, S., Dhaese, P., Zambryski, P. and Goodman, H.M. (1982) Nopaline synthase: transcript mapping and DNA sequence. *J. Mol. Appl. Genet.* 1: 561–573.

Fujii, T., Yokoyama, E.-i., Inoue, K. and Sakurai, H. (1990) The sites of electron donation of photosystem I to methyl viologen. *Biochim. Biophys. Acta* 1015: 41–48.

Genty, B., Briantais, J.-M. and Baker, N.R. (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta* 990: 87–92.

Hoagland, D.T. and Arnon, D.I. (1938) Water culture method for growing plants without soil. *Univ. Calif. Agric. Exp. Sta. Circ.* 347: 1–39.

Hodges, D.M., Andrews, C.J., Johnson, D.A. and Hamilton, R.I. (1997) Antioxidant enzyme and compound responses to chilling stress and their combining abilities in differentially sensitive maize hybrids. *Crop Sci.* 37: 857–863.

Inzé, D. and Van Montagu, M. (1995) Oxidative stress in plants. *Curr. Opin. Biotechnol.* 6: 153–158.

McKersie, B.D., Bowley, S.R., Harjanto, E. and Leprince, O. (1996) Water-deficit tolerance and field performance of transgenic alfalfa overexpressing superoxide dismutase. *Plant Physiol.* 111: 1177–1181.

McKersie, B.D., Chen, Y., de Beus, M., Bowley, S.R., Bowler, C., Inzé, D., D'Halluin, K. and Botterman, J. (1993) Superoxide dismutase enhances tolerance of freezing stress in transgenic alfalfa (*Medicago sativa* L.). *Plant Physiol.* 103: 1155–1163.

Odell, J.T., Nagy, F. and Chua, N.-H. (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* (London) 313: 810–812.

Pinhero, R.G., Rao, M.V., Paliyath, G., Murr, D.P. and Fletcher, R.A. (1997) Changes in activities of antioxidant enzymes and their relationship to genetic and paclobutrazol-induced chilling tolerance of maize seedlings. *Plant Physiol.* 114: 695–704.

Sen Gupta, A., Heinen, J.L., Holaday, A.S., Burke, J.J. and Allen, R.D. (1993a) Increased resistance to oxidative stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase. *Proc. Natl. Acad. Sci. USA* 90: 1629–1633.

Sen Gupta, A., Webb, R.P., Holaday, A.S. and Allen, R.D. (1993b) Overexpression of superoxide dismutase protects plants from oxidative stress. *Plant Physiol.* 103: 1067–1073.

Slooten, L., Capiau, K., Van Camp, W., Van Montagu, M., Sybesma, C. and Inzé, D. (1995) Factors affecting the enhancement of oxidative stress tolerance in transgenic tobacco overexpressing manganese superoxide dismutase in the chloroplasts. *Plant Physiol.* 107: 737–750.

Thompson, C.J., Rao Movva, N., Tizard, R., Crameri, R., Davies, J.E., Lauwereys, M. and Botterman, J. (1987) Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. *EMBO J.* 6: 2519–2523.

Van Breusegem, F., Kushnir, S., Slooten, L., Bauw, G., Botterman, J., Van Montagu, M. and Inzé, D. (1998) Processing of a chimeric protein in chloroplasts is different in transgenic maize and tobacco plants. *Plant Mol. Biol.* 38: 491–496.

Van Breusegem, F., Slooten, L., Stassart, J.-M., Botterman, J., Moens, T., Van Montagu, M. and Inzé, D. (1999) Effects of overproduction of tobacco MnSOD in maize chloroplasts on foliar tolerance to cold and oxidative stress. *J. Exp. Bot.* 50: 71–78.

Van Camp, W., Bowler, C., Villarroel, R., Tsang, E.W.T., Van Montagu, M. and Inzé, D. (1990) Characterization of iron superoxide dismutase cDNAs from plants obtained by genetic complementation in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 87: 9903–9907.

Van Camp, W., Capiau, K., Van Montagu, M., Inzé, D. and Slooten, L. (1996) Enhancement of oxidative stress tolerance in transgenic tobacco plants overexpressing Fe-superoxide dismutase in chloroplasts. *Plant Physiol.* 112: 1703–1714.

Van Camp, W., Van Montagu, M. and Inzé, D. (1994a) Superoxide dismutases. *In* Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. Edited by Foyer, C.H. and Mullineaux, P.M. pp. 317–341. CRC Press, Boca Raton.

Van Camp, W., Willekens, H., Bowler, C., Van Montagu, M., Inzé, D., Reupold-Popp, P., Sandermann, H., Jr. and Langebartels, C. (1994b) Elevated levels of superoxide dismutase protect transgenic plants against ozone damage. *Bio/technology* 12: 165–168.

Velten, J. and Schell, J. (1985) Selection-expression plasmid vectors for use in genetic transformation of higher plants. *Nucleic Acids Res.* 13: 6981–6998.

Winer, B.J. (1970) *Statistical Principles in Experimental Design.* International student edition. McGraw-Hill, London.

Wise, R.R. and Naylor, A.W. (1987) Chilling-enhanced photooxidation. The peroxidative destruction of lipids during chilling injury to photosynthesis and ultrastructure. *Plant Physiol.* 83: 272–277.

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