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Superoxide dismutase transgenes in sugarbeets confer resistance to oxidative agents and the fungus *C. beticola*

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Abstract

Sugarbeets carrying superoxide dismutase transgenes were developed in order to investigate the possibility of enhancing their resistance to oxidative stress. Binary T-DNA vectors carrying the chloroplastic and cytosolic superoxide dismutase genes from tomato, were used for *Agrobacterium*-mediated transformation of sugarbeet petioles. The transgenic plants were subjected to treatments known to cause oxidative stress, such as the herbicide methyl viologen and a natural photosensitizer toxin produced by the fungus *Cercospora beticola*, namely cercosporin. The transgenic plants exhibited increased tolerance to methyl viologen, to pure cercosporin, as well as to leaf infection with the fungus *C. beticola*.

Abbreviations: chl – chloroplastic; *cyt* – cytosolic; KWS – Kleinwanzlebener Saatzucht; MV – methyl viologen; *npt*II – neomycin phosphotransferase gene

Introduction

Reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide radical ($^\bullet O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl anion (OH^-), and free hydroxyl radical ($^\bullet OH$) are products of the normal cellular metabolism and cause oxidative damage to living tissues by oxidizing cellular components such as lipids, proteins, carbohydrates, and nucleic acids. Elevated levels of ROS can also arise as a result of adverse environmental conditions and chemical agents, such as heat and drought, intense light, excessively low temperatures, herbicides, and pathogens (Scandalios, 1996). An important pathogen affecting sugarbeet (*Beta vulgaris* L.) is the fungus *Cercospora beticola*, which attacks the leaves and causes leaf damage and reduced sugar content. The plant damage is brought about by a photoactivated toxin called cercosporin (Daub, 1982), which in the presence of light reacts with O_2 causing oxidative stress (Daub & Hangarter, 1983).

Protection against oxidative stress is complex and includes both enzymatic and non-enzymatic components. One of the key enzymatic systems in this defense are superoxide dismutases (SODs), which scavenge superoxide radicals and convert them into hydrogen peroxide (Bowler et al., 1992). Three different classes of SODs can be distinguished, based on their metal cofactor: copper/zinc (Cu/Zn), manganese (Mn), and

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iron (Fe). Plants generally contain Cu/ZnSOD in the cytosol, FeSOD and/or Cu/ZnSOD in the chloroplasts, and MnSOD in the mitochondria (Baum & Scandalios, 1979). SOD genes that have been isolated from various cell compartments and species include the *chloroplastic (chl)* Cu/ZnSODs from tomato (Perl-Treves et al., 1988), pea (Scioli & Zilinskas, 1988), and petunia (Tepperman et al., 1988); the *cytosolic (cyt)* Cu/ZnSOD from maize (Cannon et al., 1987), tomato (Perl-Treves et al., 1988), pea (White & Zilinskas, 1991), and *Nicotiana plumbaginifolia* (Tsang et al., 1991); the *mitochondrial (mit)* MnSOD from *N. plumbaginifolia* (Bowler et al., 1989), from maize (White & Scandalios, 1998), and yeast (Tanaka et al., 1999), as well as the *chl* FeSOD from *Arabidopsis thaliana* (Van Camp et al., 1990).

Physiological correlations between elevated SOD activity and stress tolerance have been reported (Bowler et al., 1992), suggesting that the upregulation of SOD levels may enhance the stress-defense potential of plants. Various transgenic plants that express increased amounts of SODs have been generated using the above-mentioned genes (Van Camp et al., 1990; Sen Gupta et al., 1993; McKersie et al., 1993, 1996; Trolinder & Allen, 1994; Arisi et al., 1995; Slooten et al., 1995; Payton et al., 1997; Tanaka et al., 1999; Van Breusegem et al., 1999). To investigate the possibility of improving sugarbeet tolerance to oxidative stress, two Cu/ZnSOD genes from tomato, targeted to the cytosol and the chloroplast, were introduced into sugarbeet plants via *Agrobacterium tumefaciens* transformation. While all the previous studies dealt with transgenic plant tolerance to various abiotic causes of oxidative stress, the present study shows for the first time that the SOD-transformed plants exhibited increased resistance not only to oxidative stress caused by methyl viologen (MV) but also to the fungal toxin cercosporin, as well as to leaf infection with the fungus *C. beticola*.

Materials and methods

Sugarbeet transformation

Agrobacterium-mediated plant transformation was used to produce transgenic sugarbeet plants. Petioles from the locally grown sugarbeet inbred line 028 were cocultivated with *A. tumefaciens* (Tertivanidis, 2001). Plasmids p492P31A (tomato *cyt*Cu/ZnSOD) and p492PT1 (tomato *chl*Cu/ZnSOD) (Perl-Treves et al., 1988), were introduced in the *Agrobacterium* strain LBA4404. The neomycin phosphotransferase gene, *npt*II, which confers resistance to kanamycin was also carried in the T-DNA region of all the above plasmids and was used as a selection marker.

PCR screening and Southern hybridization

Genomic DNA was isolated from leaves using the method of Dellaporta et al. (1983). Two different pairs of primer were used for PCR screening: (i) for detection of the *npt*II gene (5′ GAG GCT ATT CGG CTA TGA CTG-3′ and 5′-ATC GGG AGC GGC GAT ACC GTA-3′) and (ii) for detection of the intact *sod* transgenes, one primer for *35S* promoter of the Cauliflower Mosaic Virus (CaMV) (5′-GCT CCT ACA AAT GCC ATC A-3′) and one for *nos* terminator (5′-TTA TCC TAG TTT GCG CGC TA-3′). The 12.5 µl amplification mixtures contained 10 ng DNA, 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.4 µM of each primer, and 1.25 units of Taq DNA polymerase (Gibco-BRL, USA). Amplification was performed for 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C (Spychalla & Bevan, 1993).

For Southern blot hybridization genomic DNA was digested with EcoRI (NEB, England) and the resulting fragments were resolved on 0.8% agarose gel at 20 V for 16 h. The DNA was transferred to a nylon membrane (Amersham, England) and hybridized with DIG-labeled DNA probes either for the *npt*II gene or for the *35S* promoter that were generated using the DIG DNA labeling and detection kit (Roche).

SOD isozyme analyses

Leaf extracts from transgenic and non-transgenic sugarbeet plants were electrophoresed in non-denaturating polyacrylamide gels and negatively stained for SOD activity using a method described by Perl et al. (1993). Leaf samples (0.5–1.0 g fresh weight) were frozen in liquid nitrogen and ground to powder in precooled mortars. The powder was homogenized with extraction buffer (2 ml/g tissue) consisting of 0.2 M phosphate buffer and 2 mM EDTA (pH 7.8) as well as 14 mg/ml of isoascorbate. The resulting slurry was transferred to microfuge tubes and centrifuged (25 min, 10,000 × g, 4°C). The supernatant was assayed for protein content (Bradford, 1976) and samples (60 mg of protein per slot) were loaded on a 10% polyacrylamide gel. FeSOD and Cu/ZnSOD were inhibited using 1 mM KCN (Baum & Scandalios, 1979).

Application of MV and cercosporin

Transgenic and wild type shoots from *in vitro* culture, with six pairs of fully expanded leaves were exposed to two known oxidative agents under the conditions described below. For the MV experiments the plants were placed in 50 ml of solid MS medium (Murashige & Skoog, 1962) supplemented with 10^{-4} M MV (Sigma, USA), as described by Perl et al. (1993). The experiment was repeated three times, each time using 12 plants from each transformation event. After 16 h incubation in the dark, the shoots were transferred to fresh MS medium without MV under continuous light (45 μE m^2 s^{-1}) and observations were made after 48 h.

For the cercosporin experiments, similar shoots were placed in 50 ml solid MS medium supplemented with 40 μM cercosporin (Sigma, USA) and 5 mM methionine (Sigma, USA), as described by Daub and Hangarter (1983). The shoots were allowed to take up cercosporin from the substrate for 4 h in the dark and were subsequently exposed to light to activate the toxin. After 3 days they were transferred to fresh medium free of cercosporin and ten days later they were scored for visual symptoms.

MV-induced electrolyte leakage

Round leaf discs (6 mm in diameter) were cut with a leaf borer and were treated with a 0.5 μM aqueous solution of MV, as described by Bowler et al. (1991). Electrolyte leakage was measured with a Crison Model MicroCM 2201 conductivity meter. The amount of electrolytes due to the MV-induced oxygen radical damage was expressed as a percent of the total electrolyte content which was determined after autoclaving the samples for 5 min. For statistical analysis of the results three sets of fifteen leaf discs from each clone were used.

Fungal infections

A *C. beticola* isolate (Karaoglanidis, 2000) was grown on V-8 juice agar medium in Petri dishes with 90 mm diameter (Tuite, 1969). After 10 days of incubation under fluorescent light at 25°C sporulating colonies were rinsed with 15 ml distilled water and the conidial suspension was filtered through double-layered sterile cheesecloth and was adjusted to 8×10^3 conidia per ml. Before inoculation one droplet of 0.1% Tween 20 (Merck) was added to 100 ml of each suspension.

For each experiment, 10 plants were used from each of the transformed lines or the untransformed control, at the stage of over 10 fully expanded leaves. For the inoculation, plants were sprayed on both leaf surfaces and were kept at 25°C and saturated humidity for disease development. Susceptibility to Cercospora Leaf Spot was evaluated at 15 days. The disease severity was recorded on three leaves per plant, according to the Kleinwanzlebener Saatzucht (KWS) eight-category scale disease index (Shane & Teng, 1992), of 1–9 (1: healthy leaf, and 9: leaf and leaf stalk dead and dried up). Virulence values reported here are the mean of disease scorings on 90 leaves per line.

Results and discussion

Transgenic sugarbeet lines harboring SOD genes were developed to investigate whether they had enhanced resistance to various forms of oxidative toxicity. The method of choice was transformation via *A. tumefaciens* which has proven more efficient than other transformation methods for the sugarbeet variety used in this study (Tertivanidis, 2001). At this point it must be emphasized that no phenotypic differences were observed between transgenic and non-transgenic plants at any developmental stage. SOD proteins encoded by genes originated from tomato were targeted

Table 1. Numbers of transgenic plants obtained after *A. tumefaciens*-mediated transformation with the tomato chloroplastic or cytosolic SOD gene

Transgene	Plants selected on kanamycin	PCR positive lines	Southern positive lines
*chl*Cu/ZnSOD	17	10	5
*cyt*Cu/ZnSOD	15	9	4

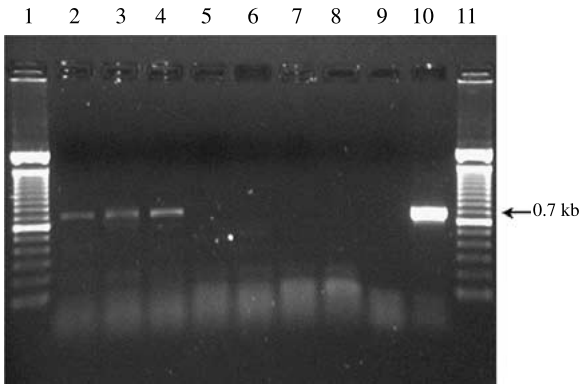

Figure 1. PCR amplification of the selection marker gene *npt*II. Lanes 1, 11: 100 bp DNA ladder; Lanes 2–4: transgenic lines with tomato *chl*Cu/ZnSOD gene; Lanes 5–7: non-transgenic lines selected on kanamycin (escapes); Lane 8: negative control (no DNA); Lane 9: negative control (DNA from untransformed plant); Lane 10: positive control (p492PT1 DNA).

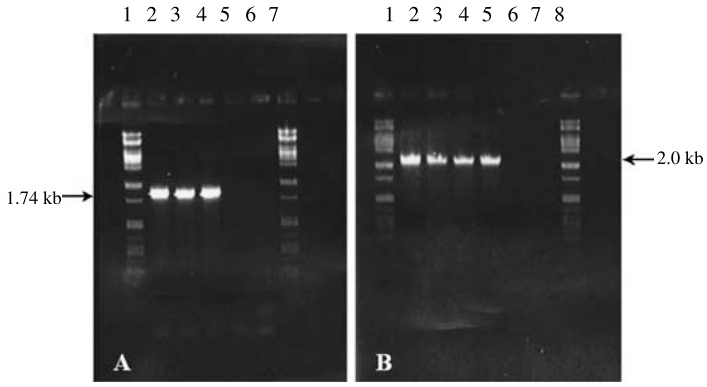

Figure 2. PCR amplification of the entire transgene with its regulatory elements (*35S* promoter, *nos* terminator). (A) Lanes 1, 7: λDNA/*Pst*I ladder; Lane 2: positive control (p492P31A DNA); Lane 3: transgenic line *cyt*Cu/ZnSOD 3; Lane 4: transgenic line *cyt*Cu/ZnSOD 4; Lane 5: negative control (no DNA); Lane 6: negative control (DNA from untransformed plant). (B) Lanes 1, 8: λDNA/*Pst*I ladder; Lane 2: positive control (p492T1 DNA); Lane 3: transgenic line *chl*Cu/ZnSOD 2; Lane 4: transgenic line *chl*Cu/ZnSOD 3; Lane 5: transgenic line *chl*Cu/ZnSOD 5; Lane 6: negative control (no DNA); Lane 7: negative control (DNA from untransformed plant).

to the cytoplasm and the chloroplast. Numbers of all transgenic plants obtained with each construct and their verification steps are summarized in Table 1.

The putative transgenic plants that had been selected for rooting on kanamycin were initially screened using PCR to eliminate plants that had escaped selection. The *npt*II gene was detected using primers that amplified a 700 bp band, identical to the band amplified from both binary plasmids that were used for transformation (Figure 1). Probes from the *npt*II or the *35S* promoter were used as probes for Southern hybridization. Since there is no information about the homology of sugarbeet and tomato SOD encoding genes it is possible that probes from tomato SOD genes might detect 'false' bands due to hybridization, to endogenous SOD genes. EcoRI-digested genomic DNA from the PCR positive lines was subjected to Southern hybridization; The 1–3 hybridization bands detected suggest a similar number of insertions (since there is no EcoRI site present in *npt*II or *35S*). Data on five *chl*Cu/ZnSOD transgenic lines and four *cyt*Cu/ZnSOD transgenic lines are presented in Figure 2. Two lines from *cyt*Cu/ZnSOD and three from *chl*Cu/ZnSOD constructs that were confirmed to possess either the *npt*II gene or the *35S* fragment were used for further experiments.

These five lines were additionally checked PCR, for presence of the entire intact construct that includes the *sod* transgenes and their promoter. For this test one pair of primer that amplifies the entire transgene with its regulatory elements (*35S* promoter, *nos* terminator) was used. The amplification products for *cyt*Cu/ZnSOD and *chl*Cu/ZnSOD transgenes were a

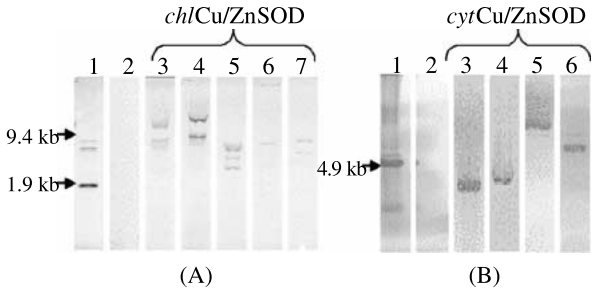

Figure 3. Southern blot analysis of transgenic sugarbeets. The genomic DNA fragments obtained after digestion with EcoRI were hybridized with a DIG-labeled: (A) *npt*II probe: Lane 1: plasmid p492PT1 partially digested with enzymes that excise the fragment used as a probe (positive control); Lane 2: untransformed plant DNA; Lanes 3–7: transgenic sugarbeets containing the tomato *chl*Cu/ZnSOD 1, 2, 3, 4, 5, respectively. (B) *35S* probe: Lane 1: linearized plasmid pART7 that contains the *35S* fragment (positive control); Lane 2: untransformed plant DNA; Lanes 3–6: transgenic sugarbeets containing the tomato *cyt*Cu/ZnSOD 1, 2, 3, 4, respectively.

1.74 kbp and a 2.00 kbp band, respectively (Figure 3). These results indicated that at least one transgene per line was intact in the sugarbeet genome.

To test expression of the transgenes leaf total protein extracts of transgenic and non-transgenic sugarbeet plants were electrophoresed in native polyacrylamide gels. The subsequent activity staining revealed three distinguishable SOD isozymes; one Cu/ZnSOD, one MnSOD, and one FeSOD. The isozymes were assigned to the gel bands after KCN inhibition. The engineered tomato Cu/ZnSOD could not be electrophoretically demarcated from the endogenous Cu/Zn isoform in the transgenic plants, but the

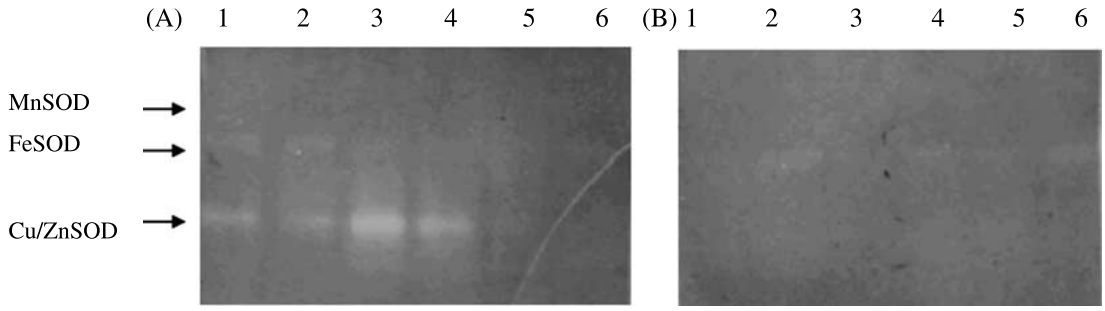

Figure 4. (A) Total proteins from leaf extracts of transgenic and non-transgenic sugarbeet shoots from *in vitro* culture, negatively stained for SOD in native polyacrylamide gels. Lanes 1–4: transgenic sugarbeets with tomato *chl*Cu/ZnSOD 2, 3 and *cyt*Cu/ZnSOD 3, 4, respectively. Lanes 5, 6: two different clones of non-transgenic sugarbeet line 028. (B) The same polyacrylamide gel as in A, stained for SOD activity after inhibition with 1 mM KCN.

Cu/Zn gel band was evidently stronger in the transgenic plants for equal amounts of loading (Figure 4).

Previous studies have correlated elevated SOD activity with increased tolerance against oxidative stress. We therefore investigated whether transgenic sugarbeet plants expressing additional SOD genes exhibited increased tolerance to superoxide radicals generated by MV. Oxidative damage was assessed visually. Shoots from two independent transformation events containing the tomato *chl*Cu/ZnSOD and *cyt*Cu/ZnSOD genes were subjected to treatment with MV. Each experiment was repeated three times and showed that after 48 h approximately 40% of the transgenic shoots exhibited obvious paraquat-related symptoms, while 85% of the control shoots collapsed and exhibited a wet-like appearance due to necrosis and destruction of cell walls (Figure 5A). MV-induced oxidative damage can also be quantitatively and statistically assessed by conductivity measurements of electrolyte leakage (Bowler et al., 1991; Sen Gupta et al., 1993; Van Camp et al., 1994). The percentage of the total electrolyte content released after treating leaf discs from one control plant and two plants of each transgenic clone with 0.5 μM MV are summarized in Figure 5B. The transgenic clones showed statistically significant differences compared to the control plants. More specifically, two *chl*Cu/ZnSOD transgenic clones differed statistically but the marginal differences among the transgenic plants did not allow any definitive conclusions about which clones were more susceptible to damage caused by MV. Previous studies (Sen Gupta et al., 1993) using transgenic tobacco plants had shown that the Cu/ZnSOD protective effect against oxidative damage was suspended at higher concentrations of MV. This was also the case in the present study, where sugarbeet leaf discs from both control and transgenic plants showed levels of electrolyte leakage close to the maximum, ranging from 92 to 100% (data not shown) when treated with higher doses of MV, namely 1.2 or 2.4 μM.

Cloned SOD genes in plants may offer protection against a vast range of oxidative agents, however the main interest of the present study was to develop locally growing sugarbeet lines with enhanced resistance against the fungus *C. beticola*. This pathogen causes serious damage in locally grown sugarbeet cultivars and lately fungus strains that are resistant to the most commonly used fungicides have arisen (Karaoglanidis, 2000). Therefore, shoots were originally subjected to treatment with the pure toxin, cercosporin. Transformed shoots harboring either the *cyt*Cu/ZnSOD or the *chl*Cu/ZnSOD as well as control shoots were allowed to absorb the fungal phytotoxin cercosporin. Under the conditions described here, 70% of the transgenic plants displayed better growth and a good overall appearance as opposed to the control plants that did not show considerable growth and showed extensive damage (Figure 6).

To examine the level of resistance against real infection with the fungus *C. beticola*, two lines of transgenic plants carrying the *cyt*Cu/ZnSOD and two lines harboring the *chl*Cu/ZnSOD were sprayed with conidial suspensions (see Materials and methods). A representative picture showing only the spotted leaves from a control plant and a transgenic *cyt*Cu/ZnSOD plant, 15 days after infection is shown in Figure 7. As shown, very few young leaves of the transgenic plant had fungal infection spots in comparison to the control plant. Statistical analysis of the scored indexes according to Duncan's multiple range test showed a significantly lower score in the four transgenic lines as compared to the non-transformed plants (Figure 8).

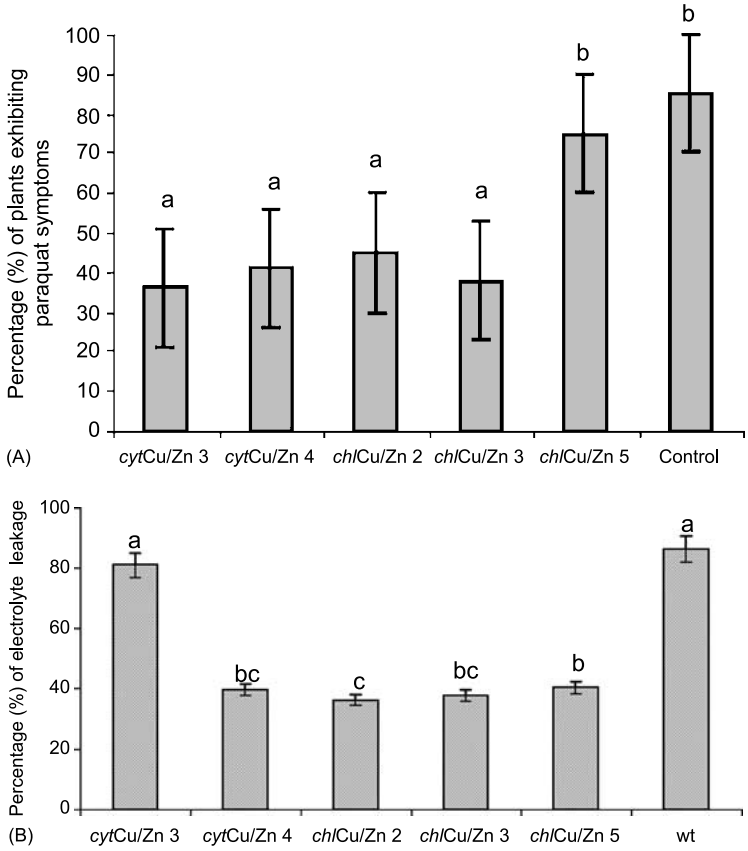

Figure 5. (A) MV-induced oxidative damage expressed as percent of shoots exhibiting obvious paraquat-related symptoms in transgenic and non-transgenic plants and the respective ±standard errors. The letters above the bars indicate the statistical differences according to LSD analysis. (*cyt*Cu/Zn 3, 4: transgenic lines harboring tomato *cyt*Cu/ZnSOD, *chl*Cu/ZnSOD 2, 3, 5: transgenic lines harboring tomato *chl*Cu/ZnSOD). (B) MV-induced electrolyte leakage expressed as percent of total electrolytes in *cyt*Cu/ZnSOD and *chl*Cu/ZnSOD transgenic plants compared to a wild type plant and the respective ±standard errors. The letters above the bars indicate the statistical differences according to LSD analysis. (*cyt*Cu/Zn 3, 4: transgenic lines harboring tomato *cyt*Cu/ZnSOD, *chl*Cu/ZnSOD 2, 3, 5: transgenic lines harboring tomato *chl*Cu/ZnSOD).

No significant differences were observed among the four transgenic lines. The apparent delay in the development of fungus-induced leaf spots in the transgenic plants may be of fundamental importance for pathogen spreading and the control of disease in the field.

The results described above indicate that the Cu/ZnSOD transgenes provide protection against the effects of 0.5 µm MV, while at higher concentrations the enzymes seem to be inactivated. Similar results were obtained in most of the previous studies, where constitutive SOD overexpression has led to increased tolerance of various plant species to oxidative stress (Bowler et al., 1991; Perl et al., 1993; McKersie et al., 1993, 1996; Sen Gupta et al., 1993; Trolinder & Allen, 1994; Van Camp et al., 1994; Tanaka et al., 1999; Van Breusegem et al., 1999). More specifically, the *chl*SOD and *cyt*SOD from tomato have rendered transgenic potato more tolerant to MV (Perl et al., 1993), while Bowler et al. (1991) found that overexpression of MnSOD enzyme conferred tolerance to cellular damage caused by oxygen radicals. In addition, the protective effect of tomato SOD transgenes in a locally growing line of sugarbeets and their potential role in the defense against the fungus *C. beticola* was investigated by toxin application *in vitro*, and by direct leaf infection under *in vivo* conditions in the greenhouse. We observed that the transgenic plants exhibited elevated resistance to both the toxin and the fungus.

Tepperman and Dunsmuir (1990) as well as Pitcher et al. (1991) found no enhanced resistance to superoxide or ozone toxicity in transgenic tobacco plants with

Figure 6. Cercosporin treatment on control (left) and transgenic (right) sugarbeet shoots containing the tomato *cyt*Cu/ZnSOD gene.

Figure 7. Visual symptoms on an individual sugarbeet plant leaves following infection with *C. beticola*, 15 days after fungal infection. Control: total number of spotted leaves from an untransformed plant, Transformed: total number of spotted leaves from a transgenic plant containing the tomato *cyt*Cu/ZnSOD gene.

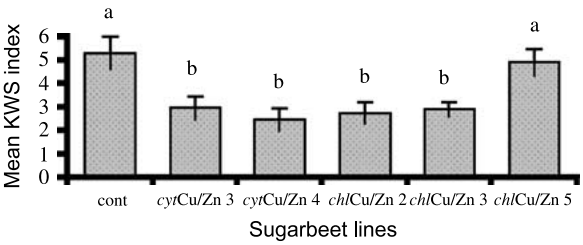

Figure 8. Development of *C. beticola*-induced spots on sugarbeet leaves 15 days after incubation with the fungus. Values are the mean KWS index scores and the respective ±standard errors. Different letters above bars denote statistically significant differences according to the Duncan's multiple range test. (*cyt*Cu/Zn 3, 4: transgenic lines harboring tomato *cyt*Cu/ZnSOD, *chl*Cu/ZnSOD 2, 3, 5: transgenic lines harboring tomato *chl*Cu/ZnSOD).

high levels of Cu/ZnSOD. These apparently divergent observations were integrated in a hypothesis proposing that the SOD protective effects depend on the superoxide radical/H_2O_2 ratio, which react to produce the more detrimental hydroxyl anion and free hydroxyl radical (Bowler et al., 1991). This critical ratio can be determined by many interacting factors, such as the type of SOD, its subcellular localization, the level of overproduction, and the endogenous H_2O_2-scavenging ability of each subcellular compartment or cell type.

Another parameter to be taken into consideration for the enhancement of the antioxidant response is that high levels of SOD activity must be complemented with simultaneous elevation of 'downstream' enzymes that quickly scavenge the resulting H_2O_2, such as catalases and peroxidases (Williamson & Scandalios, 1993). The protective role of other antioxidant genes has also been reported in breeding lines of sugarbeet, where different levels of resistance to Cercospora correlated with different levels of cytosolic peroxidases (Rautela & Payne, 1970). It is therefore evident that an enhanced antioxidant response is a result of the combined activity of enzymes such as SOD, catalases,

peroxidases, and glutathione reductases (Allen, 1995; Brisson et al., 1998). Our data indicate that the SOD transgenic plants exhibit a relative resistance to the oxidative damage caused by *C. beticola*. However, a study of the activity of different ROS scavenging enzymes and non-enzymatic factors, as well as a field study of resistance to naturally occurring infections would be needed to evaluate the stability of performance and transgene inheritance in progeny.

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