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Differential Expression of CuZn- and Fe-Superoxide Dismutase Genes of Tobacco during Development, Oxidative Stress, and Hormonal Treatments

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Chloroplasts of *Nicotiana tabacum* have two superoxide dismutases: a Fe- and a CuZn-containing enzyme, encoded by the nuclear genes *sodB* and *sodCp*, respectively. As a first step in studying the physiological function of these two enzymes, we compared the expression of *sodB* and *sodCp* in different plant organs, in response to hormonal treatments, and upon treatment with paraquat and Norflurazon. The *sodCp* transcript and active enzyme were detected only in young leaves of mature plants. The *sodB* transcript was more abundant in young compared to old leaves, but the enzymatic activity was higher in mature and senescent leaves. *sodCp* and *sodB* exhibited a different expression pattern upon treatment with abscisic acid, indole-3-acetic acid, kinetin, gibberellin, and 1-aminocyclopropane-1-carboxylate. Paraquat treatment caused a decrease in abundance of both transcripts, although the dose dependency of this decrease differed. Norflurazon-induced photooxidation resulted in a 10-fold increase of *sodCp* mRNA whereas the *sodB* transcript level was 25% higher than the control. These differences in expression might explain why both plastid-located superoxide dismutase enzymes are needed, particularly under stress conditions.

Key words: Chloroplastic superoxide dismutases — Developmental regulation of *sod* genes — Hormonal regulation of *sod* genes — *Nicotiana tabacum* — Norflurazon — Paraquat.

Active oxygen species (AOS), such as singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), are continuously produced in living cells as a by-product of normal metabolism, during metabolism of xenobiotics, and by radiation. An excess of AOS, known as oxidative stress, is harmful to cells as these molecules damage nucleic acids, proteins, and lipids (Cadenas 1989). In plants, chloroplasts are potentially the most powerful source of AOS (Asada 1994). Here, $^1O_2$ and $O_2^{\cdot-}$ are produced via direct donation of excitation energy (in the former) or electrons (in the latter) to oxygen from the photosynthetic electron chain (reviewed by Asada 1994, Foyer and Harbinson 1994). Oxidative damage in the chloroplasts is minimized by nonenzymatic antioxidants and by the combined activities of superoxide dismutases (SOD; EC 1.15.1.1) and enzymes of the ascorbate-glutathione cycle (reviewed by Foyer et al. 1994).

SOD are metalloenzymes that catalyze the dismutation of superoxide radicals to molecular oxygen and H_2O_2 (McCord and Fridovich 1969). Three classes have been defined based on their metal cofactors: copper/zinc, manganese, and an iron form (reviewed by Bowler et al. 1994, Kanematsu and Asada 1994) (for numerical designations of plant superoxide dismutases, see Zilinskas et al. 1994). Subcellular fractionation studies indicated that Mn-SOD is a mitochondrial enzyme, CuZn-SOD is predominantly cytosolic, and plastids contain Fe- and/or CuZn-SOD (Bowler et al. 1994). Several studies have reported SODs in other subcellular compartments, but no consistent pattern has yet emerged (del Río et al. 1992).

In chloroplasts of most angiosperms, the CuZn-containing form is the major SOD. cDNAs encoding chloroplast CuZn-SOD (*sodCp*) were isolated from pea (Scioli and Zilinskas 1988), *Petunia hybrida* (Tepperman et al. 1988), tomato (Perl-Treves et al. 1988), Scots pine (Karpinski et al. 1992), and spinach (Sakamoto et al. 1993). Fe-containing SOD was detected in the chloroplasts of a number of higher plant species (reviewed by Bowler et al. 1994). *sodB* cDNAs were isolated from *Nicotiana plumbaginifolia* and *Arabidopsis thaliana* (Van Camp et al. 1990), and from soybean (Crowell and Amasino 1991b). Although the significance of these two SOD isoforms has been extensively investigated by biochemical and molecular approaches, little information is available regarding co-regulation of the different chloroplast-located isoforms (Bowler et al. 1992).

In the present study, we address the question what are the specific functions of the chloroplastic CuZn-SOD and Fe-SOD in tobacco. It has been suggested that the presence of two SODs in the same organelle must have a physiological advantage (Droillard and Paulin 1990, Kwiatowski et

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; AOS, active oxygen species; IEF, isoelectric focusing; MS/2, half-strength Murashige and Skoog medium; pI, isoelectric point; SOD, superoxide dismutase; *sod*, superoxide dismutase gene and transcript; SSC, 150 mM NaCl, 15 mM Na_3-citrate, pH 7.0.

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al. 1985). This coexistence might be explained by distinct intra-organellar locations and/or different developmental and biochemical processes that they are associated with. As a first step in understanding the structure and regulation of the chloroplastic SOD system, we compared the expression patterns of *sodCp* and *sodB* during development and in response to phytohormonal and photooxidative treatments. The differential regulation of these two *sod* genes suggests a specific function for each of the enzymes in protecting chloroplasts from oxidative stress.

Materials and Methods

Plant material and growth conditions—Nicotiana tabacum cv. PBD6 was grown either in soil or on half-strength Murashige and Skoog (MS/2) medium containing 2.25 g liter^{-1} Murashige and Skoog salts (ICN Biomedicals, Costa Mesa, CA), 3% (w/v) sucrose, 2.5 mM MES (pH 5.7) and 8 g liter^{-1} agar (Difco, Detroit, MI). Unless stated otherwise, plants grown on soil were kept in a growth room at 22°C, 60% relative humidity, and 55 μmol m^{-2} s^{-1} light and those grown on MS/2 plates were kept in a controlled-environment chamber at 23°C and 50 μmol m^{-2} s^{-1} light. In both cases plants were grown in a 16-h light/8-h dark regime.

In experiments with ABA, IAA, GA_3, 1-aminocyclopropane-1-carboxylate (ACC), kinetin, and paraquat (all from Sigma, St. Louis, MO) and Norflurazon (Sandoz 9786; 4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H)-pyridazinone), 2-month-old sterile-grown plants were carefully transferred from MS/2 medium onto fresh MS/2 containing the hormone or herbicide. Norflurazon treatment was done as described in Tonkyn et al. (1992). In brief, tobacco plants grown in MS/2 medium for 2 months were transferred to either fresh MS/2 medium or MS/2 medium containing 10 μM Norflurazon. Plants were grown for an additional 20 d with a 16-h light photoperiod (100 μmol m^{-2} s^{-1} light). After the treatment, leaves of four control and four Norflurazon-treated plants were separated into an apical part (3–4 white or young leaves less than 5 cm long for the Norflurazon-treated or control plants, respectively) and a lower part (5–6 mature leaves, more than 5 cm in length), that were frozen in liquid nitrogen.

Polymerase chain reaction amplification and cloning of a sodCp cDNA fragment from N. plumbaginifolia—Total RNA was isolated from leaves using the method described by Jones et al. (1985). One microgram of total RNA was used as a template for cDNA synthesis using oligo-dT primer and the SuperScript™ Preamplification System for First Strand cDNA Synthesis (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. The resulting cDNA was used as template for PCR. Oligonucleotide primers were designed based on the tomato *sodCp* cDNA sequence (Perl-Treves et al. 1988) and the reactions were done in a PHC-2 thermocycler (Techne, Cambrige, U.K.). The PCR reaction was performed using following conditions: 94°C, 60 s; 56°C, 60 s; and 72°C, 60 s. The oligonucleotides were 5′ AATGTTGAGGGGGTTGTCACT 3′ as forward primer and 5′ ACCAACCACACCTCATGCCAA 3′ as reverse primer. The PCR product was ligated into the *Hin*cII-linearized pGEM2 vector (Promega, Madison, WI) and the construct was named pGSOD4. Plasmid DNA was sequenced according to Sanger et al. (1977).

RNA isolation and RNA gel blot analyses—Total RNA was extracted from frozen plant material as described by Logemann et al. (1987). The RNA concentration was determined by UV absorbance and samples of 12 μg were fractionated on 1.5% agarose-formaldehyde gels. Ethidium bromide was added to each sample to allow visualization of RNA under UV light for confirmation of equal sample loading. The RNA was transferred to nylon membranes (Hybond-N, Amersham, Aylesbury, U.K.) and baked at 80°C for 2 h. The [^{32}P]-labelled riboprobes used were *Nicotiana plumbaginifolia sodB* gene, pGSOD2 linearized with *Pst*I (Tsang et al. 1991) and *Nicotiana plumbaginifolia sodCp* gene, pGSOD4 linearized with *Acc*I and were synthesized using T7 RNA polymerase and Riboprobe Gemini II core system (Promega) according to the manufacturer's instructions. The blots were hybridized at 68°C following the method of Church and Gilbert (1984) and were washed at the same temperature with 2×SSC, 0.1% SDS followed by 0.2×SSC, 0.1% SDS, and finally with 0.1×SSC, 0.1% SDS. The hybridization signals were visualized after exposure to Kodak XAR-5 films, whereafter the blots were reprobed with random primed [^{32}P]-labelled *A. thaliana* 28S rRNA probe. The relative amounts of mRNA in different samples were determined by densitometric scanning of the autoradiographs using an Ultrascan Laser Densitometer (model 2202; LKB, Bromma, Sweden).

Protein extraction, superoxide dismutase, and peroxidase activity staining—Total proteins were extracted in 50 mM K-phosphate buffer (pH 7.8) containing 0.1% L-ascorbic acid and 10 mM dithiothreitol. Protein concentrations were measured using the protein assay (Bradford 1976) (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Proteins were separated by isoelectric focusing (IEF) on Ampholine® PAGplates (pH 4.0–6.5) according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). Horizontal focusing was carried out on a Multiphor II electrophoresis unit (LKB). SOD activity staining was done according to Beauchamp and Fridovich (1971). SOD isoforms were identified by H_2O_2 and KCN inhibition assays. CuZn-containing enzymes are inhibited by CN^- and inactivated by H_2O_2, whereas Fe-SOD is resistant to KCN and inhibited by H_2O_2 and Mn-SOD is resistant to both KCN and H_2O_2. For inhibition assays, gels were incubated in 50 mM K-phosphate buffer (pH 7.8), 1 mM EDTA containing either 2 mM KCN or 5 mM H_2O_2 for 30 min on room temperature prior to staining.

For guaiacol peroxidase activity staining, IEF gels were first equilibrated in 50 mM K-phosphate buffer (pH 7.8) for 30 min and then incubated in the same buffer containing 50 μg ml^{-1} 4-chloro-1-naphthol and 5 mM H_2O_2 until bands appeared.

Results and Discussion

Sequence analysis of a sodCp clone from N. plumbaginifolia—In our previous studies we have isolated cDNA clones of *N. plumbaginifolia sodA, sodB*, and *sodCc* encoding a mitochondrial Mn-SOD, chloroplastic Fe-SOD, and a cytosolic CuZn-SOD, respectively (Bowler et al. 1989, Tsang et al. 1991, Van Camp et al. 1990). Analyses of SOD isoforms from chloroplasts isolated from either *N. plumbaginifolia* (Van Camp et al. 1990) or *N. tabacum* (Slooten et al. 1995) indicated that in addition of Fe-SOD, a CuZn-containing form is present. To isolate the cDNA encoding this chloroplastic CuZn-SOD, we used a PCR-based strategy as described in Materials and Methods. The deduced amino acid sequence of the 431-bp long *sodCp* par-

tial cDNA is shown in Figure 1. The mature polypeptide region of the tobacco chloroplastic CuZn-SOD is highly homologous with those from other plant species (for example, it shared 91.5% identity at the amino acid level with tomato chloroplastic CuZn-SOD). In comparison, there is only a 68.2% amino acid identity between tobacco cytosolic and chloroplastic isoforms.

Tobacco SOD isozymes—To achieve a high resolution of SOD isoforms, we separated proteins by IEF whereafter the gels were stained for SOD activity. Differences in the staining intensity of both chloroplastic isoforms were observed when 5 μg up to 30 μg of soluble protein isolated from young leaves were loaded per lane, after which saturation of the staining reaction occurred.

The SOD isozyme pattern differed between the first apical leaf and a senescent leaf (Fig. 2). Based on the H_2O_2 and CN^- inhibition assays, two cathodic isoforms were identified as Mn-SOD and Fe-SOD, respectively. Both isoforms were more abundant in senescent leaves. The two anodic SODs detected in the extracts of young leaves were CuZn-SODs (H_2O_2 and KCN sensitive). The activity of cytosolic CuZn-SOD (identified by overexpression of the *N. plumbaginifolia sodCc* in *N. tabacum*; D. Hérouart unpublished data) decreased with increasing leaf age. Chloroplastic CuZn-SOD isoform was not detectable in protein extracts of senescent leaves. Two additional bands of SOD-like activity, detected in protein extracts of senescent leaves, appeared to be nitroblue tetrazolium oxidases. These enzymes had guaiacol peroxidase activity as was confirmed by staining the IEF gel with 4-chloro-1-naphthol. These two acidic peroxidase isoforms were more abundant in senescent tissues and had an isoelectric point (pI) identical to that of the nitroblue tetrazolium oxidase detected after the SOD activity staining (Fig. 2).

Both plastid-located SODs are developmentally regulated—Most *sod* genes studied to date have been shown to be developmentally regulated. The *sodCp* transcript level was high in tomato shoot tips and decreased in fully expanded leaves (Perl-Treves and Galun 1991). A similar distribution pattern of the *sodCp* transcript was reported in pea (Strid 1993). The *sodB* mRNA was found to accumulate during leaf expansion in soybean (Crowell and Amasino 1991a).

In this study, the steady-state levels of *sodCp* and *sodB* transcripts were determined in leaves of different age and in stems and roots of mature non-flowering

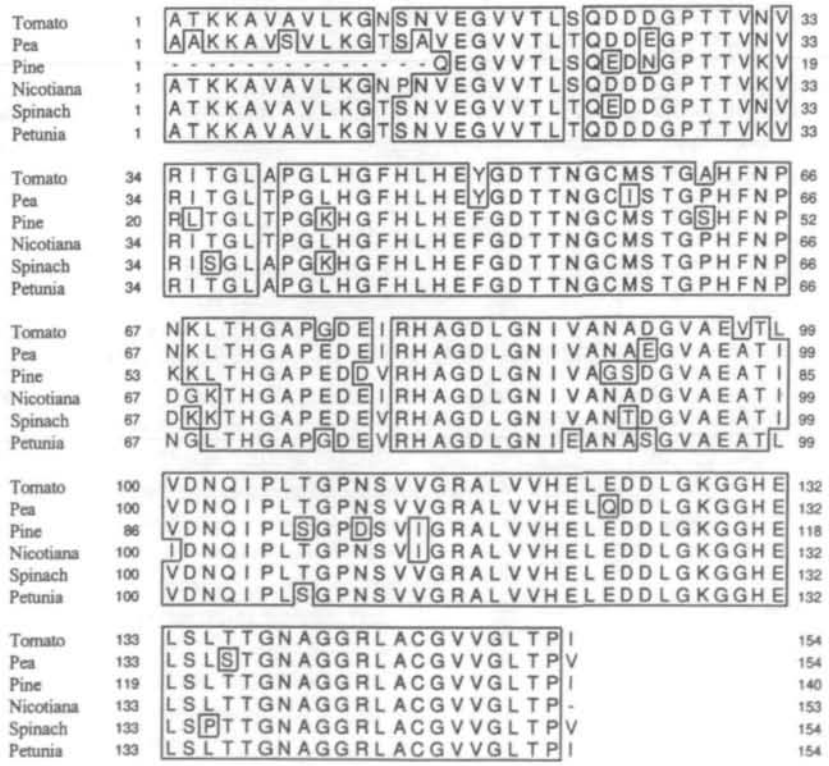

Fig. 1 Alignment of the predicted amino acid sequences of *N. plumbaginifolia* chloroplastic CuZn-SOD and other chloroplastic CuZn-SOD mature proteins contained in the EMBL Database. Amino acid residues are numbered starting from the first amino acid of the predicted mature polypeptide region (Sakamoto et al. 1993). Sequences are derived from tomato (Perl-Treves et al. 1988), pea (Scioli and Zilinskas 1988), Scots pine (Karpinski et al. 1992), *N. plumbaginifolia* (this article), spinach (Sakamoto et al. 1993), and *Petunia hybrida* (Tepperman et al. 1988).

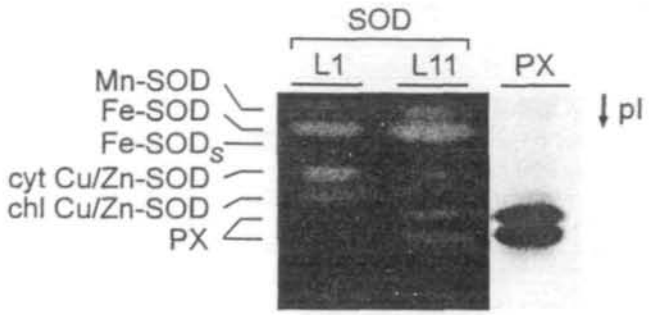

Fig. 2 Comparison of the SOD isoform profiles from the first leaf (L1) and the eleventh leaf (L11) of a mature non-flowering plant. IEF gel (pH 4.0–6.5; 30 μg protein per lane) was stained for SOD activity. Different SOD isoforms (chl, chloroplastic; cyt, cytosolic; Fe-SOD$_s$, senescence-associated Fe-SOD) and peroxidases (PX) are indicated. IEF gels were stained for peroxidase activity with 4-chloro-1-naphthol as described in Materials and Methods.

N. tabacum (Fig. 3). The *sodCp* mRNA was 4-fold more abundant in the youngest leaf (L1) compared to the second leaf (L2) and decreased sharply to an undetectable level in fully expanded leaves. The *sodB* mRNA level was also higher in young leaves (80% higher compared to the second leaf), but the transcript remained detectable in mature and senescing leaves. Steady-state levels of both transcripts in different regions of the stem (that is, younger vs. older parts) resembled the pattern found in the leaves. Both transcripts were more abundant in the upper part of the stem, encompassing leaves one to four. The *sodCp* mRNA was not detected in the lower parts of the stem, whereas the level of the *sodB* transcript decreased, but remained detectable in older parts of the stem. As expected, both mRNAs

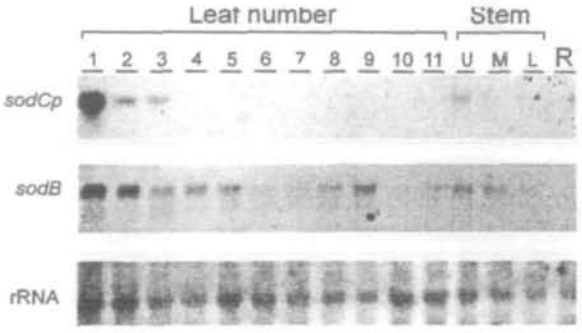

Fig. 3 Comparison of *sodCp* and *sodB* mRNA distribution in tobacco organs. Representative results from RNA gel blot analysis (12 μg total RNA per lane) of *sodCp* and *sodB* expression are shown. Total RNA was isolated from leaves (leaf 1 being the first apical leaf larger than 9 cm, and leaf 11 being a senescent leaf), stems, and roots (R). Stems were separated into three segments: uppermost (U) containing the apical bud and the stem until leaf 4, the middle part (M) until leaf 9, and the lowest part (L) corresponding to the tenth and the eleventh leaves.

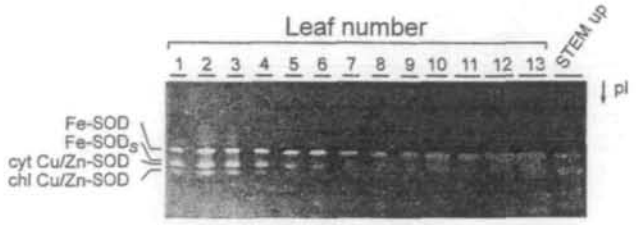

Fig. 4 Distribution of SOD isozymes during leaf expansion and senescence. The leaf numbering is the same as in Fig. 3 and STEM$_{up}$ denotes the uppermost part of the stem. The amount of protein loaded per lane was 15 μg.

could not be detected in the roots.

Figure 4 illustrates the changes in SOD isozyme patterns during leaf maturation. The chloroplastic CuZn-SOD activity levels reflected the gradient of *sodCp* mRNA: the highest activity was found in young leaves and a gradual decrease was observed with leaf age. There was no direct correlation between *sodB* mRNA abundance and Fe-SOD activity: while the *sodB* transcript level decreased, the Fe-SOD activity increased with leaf age. These data suggest that Fe-SOD has a high turnover in young leaves and/or is accumulating in older leaves. An additional Fe-SOD isoform with lower pI (Fe-SOD$_s$) was detected in mature and senescent leaves. Parallel to the decreased activities of chloroplastic and cytosolic CuZn-SOD isoforms (from the seventh leaf on), an increased activity of Fe-SOD$_s$ was evident. The isoelectric points of Fe-SOD$_s$ and cytosolic CuZn-SOD are similar, which can result in an insufficient resolution of these isoforms in senescent leaves. Analysis of the SOD patterns of the oldest leaf and the apical part of the stem showed that in senescent leaves the major isoforms were Fe-SOD and Fe-SOD$_s$ (Fig. 4). The existence of age-related SOD isoforms, including multiple Fe-SOD isoforms with close pI values, has previously been reported in carnations (Droillard and Paulin 1990).

Thus, the two chloroplastic *sod* genes are differently regulated during development. The presence of the *sodCp* transcript and corresponding enzyme in young leaves only, in contrast with the Fe-SOD that is present in all photosynthetic tissues, is the first lead to resolve their specific functions.

Paraquat-induced oxidative stress results in distinctive responses of sodCp and sodB genes—In the presence of light, paraquat causes formation of $O_2^{\cdot -}$ preferentially within the chloroplasts (Halliwell and Gutteridge 1989). It was reported that overproduction of either Mn-SOD (Bowler et al. 1991, Slooten et al. 1995) or chloroplastic CuZn-SOD (Sen Gupta et al. 1993) in tobacco chloroplasts resulted into an increased resistance to paraquat-mediated damage. In addition, it was demonstrated that in some paraquat-resistant weed biotypes chloroplastic SOD activity is elevated (reviewed in Hart and DiTomaso 1994).

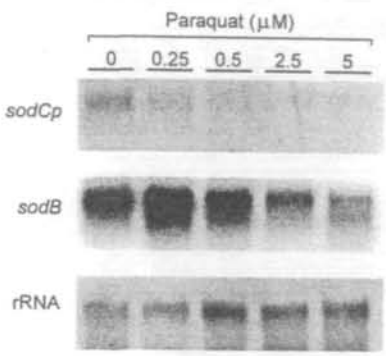

Fig. 5 Effects of paraquat on steady-state mRNA levels of *sodCp* and *sodB*. Tobacco plants were grown on MS/2 medium with the indicated concentrations of paraquat for 4 d. The aerial parts of four plants (apex, 3 to 4 young leaves, and 4 to 5 mature leaves) were pooled for each treatment and total RNA was extracted. A single RNA gel blot was hybridized to the *sodCp* probe and re-hybridized sequentially to *sodB* and ribosomal RNA (rRNA).

We examined the expression of *sodCp* and *sodB* genes after a treatment with a range of paraquat doses. Two-month-old plants grown in sterile conditions were transferred to media containing 0.25, 0.5, 2.5, and 5 μM paraquat and treated for 4 d. In the light conditions used, only plants grown on 5 μM paraquat became chlorotic. The accumulation patterns of *sodCp* and *sodB* mRNAs are shown in Figure 5. The level of *sodCp* mRNA was reduced to 20% of the control level in plants grown on 0.25 μM paraquat and to approximately 10% in plants grown on 2.5 μM paraquat. In plants grown on 0.25 μM paraquat, the *sodB* transcript level was on average 20% higher than in the control, but higher paraquat concentrations resulted in a gradual decrease of *sodB* mRNA abundance (by 50% on 0.5 μM, 70% on 2.5 μM and 85% on 5 μM paraquat). Chloroplastic CuZn-SOD activity was not detected in plants grown on paraquat, and the Fe-SOD activity levels were reduced at higher paraquat concentrations (data not shown).

Paraquat-mediated and seemingly dose-independent reduction of *sodCp* mRNA level is not an isolated case in which the accumulation of the transcript for this protective enzyme is greatly reduced after stress. In pea, 24 h after UV-B treatment the level of *sodCp* mRNA is reduced to 10% of the level in the control plants (Strid 1993). The comparison of *sodCp* and *sodB* expression profiles would suggest that Fe-SOD is a more important protectant against paraquat-induced oxidative stress in the chloroplast. Interestingly, the $O_2^{\cdot-}$ dismutation product H_2O_2 is not inactivating CuZn-SOD and Fe-SOD to the same extent (reviewed by Kanematsu and Asada 1994). CuZn-SOD is completely inactivated by H_2O_2 whereas Fe-SOD is inactivated to a limit of 90% (Kanematsu and Asada 1994). This residual H_2O_2-resistant activity may play an important role during stress.

Photooxidative destruction of chloroplasts exerts differential effects on the expression of sodCp and sodB—To examine whether the expression of plastid-located *sod* genes is dependent on functional chloroplast, we analyzed their expression under photobleaching conditions caused by a Norflurazon treatment. This herbicide blocks de novo synthesis of carotenoids by inhibiting phytoene desaturase, the rate-limiting enzyme in carotenoid biosynthesis. One of the most important processes involving carotenoids in photosynthetic systems is the quenching of triplet-state chlorophyll, thereby preventing the generation of singlet oxygen (Pallett and Joung 1993). We analyzed the levels of plastid-located *sod* transcripts in plants grown for 2 months on MS/2 medium that were transferred to either MS/2 medium or 10 μM Norflurazon-containing MS/2 medium and grown for an additional 20 d in light (100 $\mu mol\ m^{-2}\ s^{-1}$). By the time the leaves were harvested, 3–4 apical leaves (less than 5 cm in length) of the Norflurazon-treated plants appeared completely white, whereas the older leaves (more than 5 cm in length) were lighter green than the control plants indicating photooxidative damage. Analysis of total protein extracts from white leaves by SDS/polyacrylamide gel electrophoresis revealed the absence of the small and large subunit of ribulose-1,5-bisphosphate carboxylase (data not shown). The chlorophyll *a/b*-binding protein (*cab*) mRNA level was 3-fold higher in the young leaves of the control plants compared to the white leaves of the Norflurazon-treated plants (data not shown). The level of the *sodCp* transcript was approximately 10-fold higher in apical (white) leaves from Norflurazon-treated plants compared to that of young leaves at the same age of control plants (Fig. 6). The *sodB* mRNA level in apical photobleached

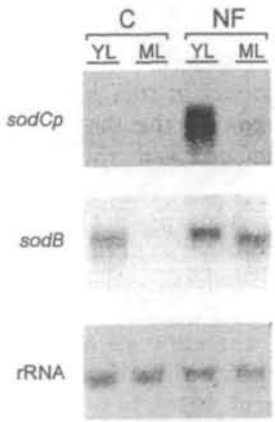

Fig. 6 Effects of Norflurazon on plastid-located *sod* genes. Two month-old plants were grown for an additional 20 d on MS/2 media in the absence (C) and the presence of 10 μM Norflurazon (NF). Total RNA was extracted from young (YL; 3 to 4 apical leaves less than 5 cm long) and mature leaves (ML; from the fifth leaf on). Autoradiograms of the representative RNA gel blot hybridization experiments are shown.

leaves from Norflurazon-treated plants was 25% higher compared to that of the control. Interestingly, the naturally occurring gradient of *sodB* mRNA, i.e. higher transcript levels in young compared to old leaves, was abolished in Norflurazon-treated plants (Fig 6). We cannot rule out the possibility that the increase of the *sodB* mRNA level in green leaves of the treated plants is due to some cause other than photooxidative damage. No changes of the SOD isozyme levels were detected upon this treatment (data not shown). We can speculate that the level of active enzyme did not change, albeit a higher mRNA level due to a higher rate of enzyme turnover in the stressed tissue (Williamson and Scandalios 1992).

Studies on seedlings lacking carotenoids showed that photooxidation inhibited the transcription of a set of nuclear genes (Mayfield and Taylor 1984, 1987, Oelmüller and Mohr 1986, Oelmüller et al. 1986). It was proposed that a plastid-born signal, which is sensitive to the developmental and/or functional state of the chloroplast, is necessary for the optimal transcription of a set of nuclear-encoded chloroplast proteins (reviewed by Taylor 1989, Mayfield 1990). There are several explanations for the high level of *sodCp* mRNA in white leaves. Because carotenoids are the precursors of ABA (reviewed by Taylor 1991, Zeevaart et al. 1991), the high expression levels can be a result of ABA deficiency (see below). Alternatively, it can be speculated that a signal produced in mature chloroplasts represses *sodCp* gene expression. Therefore, the lack of this signal from photooxidised plastids can result in a de-repression of this gene. Another possible explanation is that a signal from photobleached plastids might induce the expression of the *sodCp* gene. Additional experiments could help to distinguish between these possibilities. The steady-state level of *sodB* in the photobleached tissue increased by 25%, suggesting that for its expression *sodB* does not require functional chloroplasts.

Role of hormones in sodCp and sodB expression— The results above confirm that the plastid *sod* genes are developmentally regulated and transcriptionally controlled by stress. Previous reports have revealed some effects of hormones on *sodCp* and *sodB* expression (Casano et al. 1994, Crowell and Amasino 1991a, Perl-Treves and Galun 1991). The level of *sodB* increased during auxin and cytokinin starvation in soybean cell cultures (Crowell and Amasino 1991a), whereas *sodB* mRNA levels did not increase after dark-adapted barley leaves were treated with kinetin (Casano et al. 1994). In tomato plants treated with ethephon, *sodCp* mRNA and the chloroplastic CuZn-SOD levels increased (Perl-Treves and Galun 1991).

To obtain a more general view on the effects of plant hormones on *sodCp* and *sodB* expression, we analyzed their transcriptional response to IAA, ABA, GA_3, kinetin, and the ethylene precursor ACC. We characterized the effects of a 24-h-long treatment with a single dose of the classical phytohormones using 2-month-old sterile-grown plants in which both transcripts are present at a relatively high level (Fig. 7). In all cases, the Fe-SOD and chloroplastic CuZn-SOD activity levels were comparable to the control after 24 h of treatment (data not shown). The discrepancy between hormone-induced decreases or increases of *sodCp* and *sodB* transcript levels and the constant activity of the corresponding enzymes suggest that SOD activity is regulated post-transcriptionally, for example, by regulating efficiency of translation and/or protein stability.

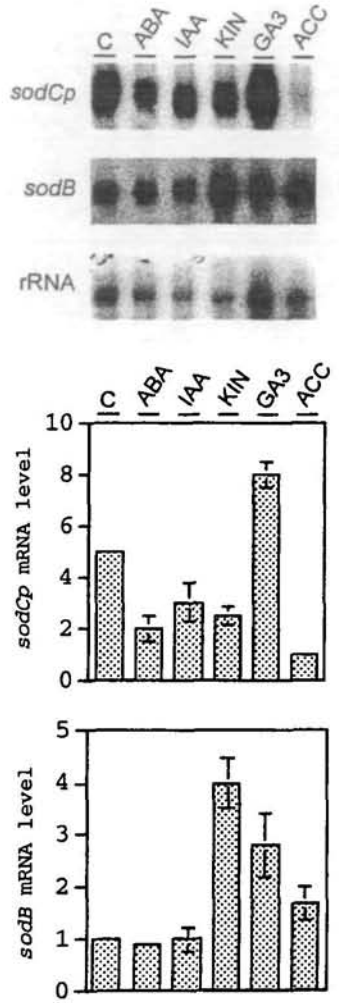

Fig. 7 Effects of hormone treatments on *sodCp* and *sodB* mRNA levels. (A) Two-month-old sterile-grown tobacco plants were transferred to MS/2 medium containing either 10 μM ABA, 10 μM IAA, 10 μM kinetin, 5 μM GA_3, or 200 μM ACC. After 24 h of growth, aerial parts (apex, 3 to 4 young leaves, and 4 to 5 mature leaves) were collected and total RNA was extracted. Representative data from RNA gel blot hybridization analysis are shown. (B) Densitometric data are presented as mean ± SEM of two measurements. The value of 1 on the ordinate was given to the mRNA level of the non-treated and ACC-treated plants in the case of *sodB* and *sodCp*, respectively.

The *sodCp* mRNA levels decreased in response to all the hormone treatments (by 60% for ABA, by 50% for IAA and kinetin, and by 80% for ACC), except for the GA_3 treatment that resulted in an induction of approximately 2-fold. Both *sodCp* and *sodB* genes showed a similar response to the GA_3 treatment. ABA and IAA had no effects on *sodB* expression and the *sodB* mRNA level was higher in response to kinetin (3-fold) and ACC (1.5-fold).

These differences in the expression pattern again indicate different functions for *sodCp* and *sodB* in the chloroplastic antioxidative system. The ACC-mediated repression of *sodCp* can relate to the low expression level of this gene in senescing tissues. The IAA-mediated repression of this gene might be associated with ethylene biosynthesis, because plants have been shown to have an IAA-induced ACC synthase activity (Abel et al. 1995). It is puzzling that *sodCp*, which based on its developmental expression pattern would be specific for young tissue, is repressed by the anti-senescence hormone kinetin. Transcription of some members of *sod* multigene families, such as rice *sodCc*-2 (Sakamoto et al. 1995) and maize *sod3.2*, *sod3.3*, and *sod3.4* (Zhu and Scandalios 1994) is induced by ABA. To our knowledge, this is the first report of an ABA-mediated decrease in *sodC* transcript level. The finding that *sodB* is induced by kinetin, as well as by ACC is somewhat puzzling, given the opposing effects that are ascribed to cytokinins and ethylene (Abeles et al. 1992). On the other hand, this result can suggest a role for ethylene in mediating senescence-related changes of *sodB* expression.

Concluding remarks

There are a considerable number of unresolved questions on how the expression of *sod* genes is regulated in plant cells. That is particularly true for plastid-located *sod* genes. Next to questions such as how oxidative stress is sensed, transduced, and transmitted and how the response of antioxidative genes is coordinated, we also need to analyze why some plant species contain multiple SOD isoforms in the chloroplast, how is their expression influenced by chloroplast biogenesis and functional state, and what are their specific functions. We analyzed the expression of *sodCp* and *sodB* in tobacco to establish the differences in their regulation. Our data suggest that chloroplastic Fe-SOD and CuZn-SOD could be involved in protecting different parts and/or processes within this organelle. CuZn-SOD in spinach was shown to be associated with the PSI complex in thylakoids, and not with the putative superoxide radical generation site in the stroma (Ogawa et al. 1995). The compartmentalization of CuZn-SOD on the stroma-facing thylakoids suggests the prompt scavenging of the superoxide radical near the site of production (Ogawa et al. 1995). Thus, further studies, combined with the analysis of the intra-organellar distribution of CuZn- and Fe-SOD in tobacco, should lead to a better understanding of the nature of protective functions of these two enzymes and of the complexity of the signaling pathways which mediate such a fine regulation of the chloroplastic SOD system.

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References

Abel, S., Nguyen, M.D., Chow, W. and Theologis, A. (1995) *ASC4*, a primary indoleacetic acid-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase in *Arabidopsis thaliana*. Structural characterization, expression in *Escherichia coli*, and expression characteristics in response to auxin. *J. Biol. Chem.* 270: 19093–19099.

Abeles, F.B., Morgan, P.W. and Saltveit, M.E. (1992) Regulation of ethylene production by internal, environmental and stress factors. *In* Ethylene in Plant Biology, 2nd ed. Edited by Abeles, F.B., Morgan, P.W. and Saltveit, M.E. pp. 56–111. Academic Press, San Diego.

Asada, K. (1994) Production and action of active oxygen species in photosynthetic tissues. *In* Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. Edited by Foyer, C.H. and Mullineaux, P.M. pp. 77–104. CRC Press, Boca Raton.

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., Alliotte, T., De Loose, M., Van Montagu, M. and Inzé, D. (1989) The induction of manganese superoxide dismutase in response to stress in *Nicotiana plumbaginifolia*. *EMBO J.* 8: 31–38.

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.

Bowler, C., Van Montagu, M. and Inzé, D. (1992) Superoxide dismutase and stress tolerance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 43: 83–116.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.

Cadenas, E. (1989) Biochemistry of oxygen toxicity. *Annu. Rev. Biochem.* 58: 79–110.

Casano, L.M., Martín, M. and Sabater, B. (1994) Sensitivity of superoxide dismutase transcript levels and activities to oxidative stress is lower in mature-senescent than in young barley leaves. *Plant Physiol.* 106: 1033–1039.

Church, G.M. and Gilbert, W. (1984) Genomic sequencing. *Proc. Natl. Acad. Sci. USA* 81: 1991–1995.

Crowell, D.N. and Amasino, R.M. (1991a) Induction of specific mRNAs in cultured soybean cells during cytokinin or auxin starvation. *Plant Physiol.* 95: 711–715.

Crowell, D.N. and Amasino, R.M. (1991b) Nucleotide sequence of an iron superoxide dismutase complementary DNA from soybean. *Plant Physiol.* 96: 1393–1394.

del Río, L.A., Sandalio, L.M., Palma, J.M., Bueno, P. and Corpas, F.J.

(1992) Metabolism of oxygen radicals in peroxisomes and cellular implications. *Free Rad. Biol. Med.* 13: 557–580.

Droillard, M.-J. and Paulin, A. (1990) Isozymes of superoxide dismutase in mitochondria and peroxisomes isolated from petals of carnation (*Dianthus caryophyllus*) during senescence. *Plant Physiol.* 94: 1187–1192.

Foyer, C.H., Descourvières, P. and Kunert, K.J. (1994) Protection against oxygen radicals: an important defence mechanism studied in transgenic plants. *Plant Cell Environ.* 17: 507–523.

Foyer, C.H. and Harbinson, J. (1994) Oxygen metabolism and the regulation of photosynthetic electron transport. *In* Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. Edited by Foyer, C.H. and Mullineaux, P.M. pp. 1–42. CRC Press, Boca Raton.

Halliwell, B. and Gutteridge, J.M.C. (1989) *Free Radicals in Biology and Medicine.* Clarendon Press, Oxford.

Hart, J.J. and DiTomaso, J.M. (1994) Sequestration and oxygen radical detoxification as mechanisms of paraquat resistance. *Weed Sci.* 42: 277–284.

Jones, J.D.G., Dunsmuir, P. and Bedbrook, J. (1985) High level expression of introduced chimaeric genes in regenerated transformed plants. *EMBO J.* 4: 2411–2418.

Kanematsu, S. and Asada, K. (1990) Characteristic amino acid sequences of chloroplast and cytosol isozymes of CuZn-superoxide dismutase in spinach, rice and horsetail. *Plant Cell Physiol.* 31: 99–112.

Kanematsu, S. and Asada, K. (1994) Superoxide dismutase. *In* Molecular Aspects of Enzyme Catalysis. Edited by Fukui, T. and Soda, K. pp. 191–210. Kodansha Ltd., Tokyo.

Karpinski, S., Wingsle, G., Olsson, O. and Hällgren, J.-E. (1992) Characterization of cDNAs encoding CuZn-superoxide dismutases in Scots pine. *Plant Mol. Biol.* 18: 545–555.

Kwiatowski, J., Safianowska, A. and Kaniuga, Z. (1985) Isolation and characterization of an iron-containing superoxide dismutase from tomato leaves, *Lycopersicon esculentum. Eur. J. Biochem.* 146: 459–466.

Logemann, J., Schell, J. and Willmitzer, L. (1987) Improved method for the isolation of RNA from plant tissues. *Anal. Biochem.* 163: 16–20.

Mayfield, S.P. (1990) Chloroplast gene regulation: interaction of the nuclear and chloroplast genomes in the expression of photosynthetic proteins. *Curr. Opin. Cell Biol.* 2: 509–513.

Mayfield, S.P. and Taylor, W.C. (1984) Carotenoid-deficient maize seedlings fail to accumulate light-harvesting chlorophyll *a/b* binding protein (LHCP) mRNA. *Eur. J. Biochem.* 144: 79–84.

Mayfield, S.P. and Taylor, W.C. (1987) Chloroplast photooxidation inhibits the expression of a set of nuclear genes. *Mol. Gen. Genet.* 208: 309–314.

McCord, J.M. and Fridovich, I. (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* 244: 6049–6055.

Oelmüller, R., Levitan, I., Bergfeld, R., Rajasekhar, V.K. and Mohr, H. (1986) Expression of nuclear genes as affected by treatments acting on the plastids. *Planta* 168: 482–492.

Oelmüller, R. and Mohr, H. (1986) Photooxidative destruction of chloroplasts and its consequences for expression of nuclear genes. *Planta* 167: 106–113.

Ogawa, K., Kanematsu, S., Takabe, K. and Asada, K. (1995) Attachment of CuZn-superoxide dismutase to thylakoid membranes at the site of superoxide generation (PSI) in spinach chloroplasts: detection by immunogold labeling after rapid freezing and substitution method. *Plant Cell Physiol.* 36: 565–573.

Pallett, K.E. and Young, A.J. (1993) Carotenoids. *In* Antioxidants in Higher Plants. Edited by Alscher, R.G. and Hess, J.L. pp. 59–89. CRC Press, Boca Raton.

Perl-Treves, R. and Galun, E. (1991) The tomato Cu,Zn superoxide dismutase genes are developmentally regulated and respond to light and stress. *Plant Mol. Biol.* 17: 745–760.

Perl-Treves, R., Nacmias, B., Aviv, D., Zeelon, E.P. and Galun, E. (1988) Isolation of two cDNA clones from tomato containing two different superoxide dismutase sequences. *Plant Mol. Biol.* 11: 609–623.

Sakamoto, A., Ohsuga, H., Wakaura, M., Mitsukawa, N., Hibino, T., Masumura, T., Sasaki, Y. and Tanaka, K. (1993) cDNA cloning and expression of the plastidic copper/zinc-superoxide dismutase from spinach (*Spinacia oleracea* L.) leaves. *Plant Cell Physiol.* 34: 965–968.

Sakamoto, A., Okumura, T., Kaminaka, H., Sumi, K. and Tanaka, K. (1995) Structure and differential response to abscisic acid of two promoters for the cytosolic copper/zinc-superoxide dismutase genes, *SodCc1* and *SodCc2*, in rice protoplasts. *FEBS Lett.* 358: 62–66.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74: 5463–5467.

Scioli, J.R. and Zilinskas, B.A. (1988) Cloning and characterization of a cDNA encoding the chloroplastic copper/zinc-superoxide dismutase from pea. *Proc. Natl. Acad. Sci. USA* 85: 7661–7665.

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

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.

Strid, Å. (1993) Alteration in expression of defence genes in *Pisum sativum* after exposure to supplementary ultraviolet-B radiation. *Plant Cell Physiol.* 34: 949–953.

Taylor, I.B. (1991) Genetics of ABA synthesis. *In* Abscisic Acid: Physiology and Biochemistry, (Environmental Plant Biology Series). Edited by Davies, W.J. and Jones, H.G. pp. 23–35. Bios Scientific Publishers, Oxford.

Taylor, W.C. (1989) Regulatory interactions between nuclear and plastid genomes. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40: 211–233.

Tepperman, J., Katayama, C. and Dunsmuir, P. (1988) Cloning and nucleotide sequence of a petunia gene encoding a chloroplast-localized superoxide dismutase. *Plant Mol. Biol.* 11: 871–872.

Tonkyn, J.C., Deng, X.-W. and Gruissem, W. (1992) Regulation of plastid gene expression during photooxidative stress. *Plant Physiol.* 99: 1406–1415.

Tsang, E.W.T., Bowler, C., Hérouart, D., Van Camp, W., Villarroel, R., Genetello, C., Van Montagu, M. and Inzé, D. (1991) Differential regulation of superoxide dismutases in plants exposed to environmental stress. *Plant Cell* 3: 783–792.

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.

Williamson, J.D. and Scandalios, J.G. (1992) Differential response of maize catalases and superoxide dismutases to the photoactivated fungal toxin cercosporin. *Plant J.* 2: 351–358.

Zeevaart, J.A.D., Rock, C.D., Fantauzzo, F., Heath, T.G. and Gage, D.A. (1991) Metabolism of ABA and its physiological implications. *In* Abscisic Acid: Physiology and Biochemistry, (Environmental Plant Biology Series). Edited by Davies, W.J. and Jones, H.G. pp. 39–52. Bios Scientific Publishers, Oxford.

Zhu, D. and Scandalios, J.G. (1994) Differential accumulation of manganese-superoxide dismutase transcripts in maize in response to abscisic acid and high osmoticum. *Plant Physiol.* 106: 173–178.

Zilinskas, B.A., Asada, K., Galun, E., Inzé, D. and Tanaka, K. (1994) Genes encoding superoxide dismutases. *Plant Mol. Biol. Reporter (CPGN Supp.)* 12: S73–S74.

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