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Genetic Engineering of Oxidative Stress Resistance in Higher Plants

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Genetic engineering of oxidative stress resistance in higher plants

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SUMMARY

Many environmental conditions induce an oxidative stress in plant cells by the generation of abnormal concentrations of reactive-oxygen intermediates (ROI) such as superoxide radicals, hydrogen peroxide and hydroxyl radicals. To protect themselves against these potentially damaging molecules, plants have evolved several enzymic mechanisms including superoxide dismutases (SODs), peroxidases and catalases. Recent molecular studies in *Nicotiana* species showed that the genes coding for the different ROI-scavenging enzymes are differentially regulated in response to environmental stress. Transgenic plants which overexpressed SOD genes in chloroplasts are more tolerant to treatment with the superoxide-generating herbicide paraquat and to ozone fumigation. Strategies to engineer plants with high resistance to oxidative stress are discussed.

1. INTRODUCTION

All aerobic organisms produce, as an unavoidable consequence of their oxygen consumption, reactive-oxygen intermediates (ROI) such as singlet oxygen (O_2^1), superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals (OH°), and hydrogen peroxide (H_2O_2) (Halliwell & Gutteridge 1989; Basaga 1990). An oxidative stress situation has been defined as an alteration of the steady-state concentrations of components of cellular redox systems in favour of the oxidized form (Sies 1985). Numerous environmentally adverse conditions such as hyperoxia, radiation, heat shock, chilling, air pollutants (e.g. ozone, sulphur dioxide), herbicides (e.g. paraquat), and pathogens (e.g. *Cercospora*) are known to mediate, at least in part, cellular damage by the enhanced production of ROI (Bowler *et al.* 1991; Scandalios 1993).

The mechanisms of protection against ROI may be more efficient in plants compared to other eukaryotes because plants not only consume O_2 during respiration but they also generate it during photosynthesis. To prevent oxidation of cellular components, plant cells maintain low steady-state levels of ROI by a variety of non-enzymic and enzymic antioxidant mechanisms (Asada & Takahashi 1987; Larson 1988).

Superoxide radicals ($O_2^{\cdot-}$) are rapidly converted into H_2O_2 by superoxide dismutases (SODs), which are metalloenzymes containing either manganese (Mn), iron (Fe), or copper/zinc (Cu/Zn) as their prosthetic metal (figure 1*b*). Plants usually contain a cytosolic Cu/ZnSOD, a mitochondrial MnSOD, and a chloroplastic Cu/ZnSOD and/or FeSOD (Bowler *et al.* 1992). Removal of H_2O_2 is also very critical since this molecule reacts spontaneously with $O_2^{\cdot-}$ to form the highly reactive hydroxyl radical (Fenton reaction; figure 1*a*).

Catalases (CATs) scavenge H_2O_2 in peroxisomes (figure 1*c*) and various peroxidases serve this function in other cellular compartments. In plants, it is generally accepted that ascorbate peroxidase (APx) is the major H_2O_2 scavenger. APx is part of the ascorbate-glutathione cycle (figure 1*d*) which was originally discovered in chloroplasts (Foyer & Halliwell 1976) but may operate in the cytosol as well. In animals, glutathione peroxidase (GPx) is the major H_2O_2 scavenger in the cytosol and mitochondria (figure 1*e*) and its occurrence in plants has been debated for a long time. However, recently, the isolation of cDNAs with high homology to glutathione peroxidases in *Nicotiana sylvestris* (Criqui *et al.* 1992) and *Citrus* (Holland *et al.* 1993) suggests that a functional glutathione cycle also exists in plants. The subcellular localization of these GPx in plants has still to be established.

Here, we report on recent molecular studies on plant ROI-scavenging enzymes and their relevance to engineering oxidative stress-tolerant plants.

2. TRANSCRIPTIONAL ACTIVATION OF STRESS-RESPONSIVE GENES

Before considering a strategy for engineering stress-tolerant plants, it is necessary to characterize the

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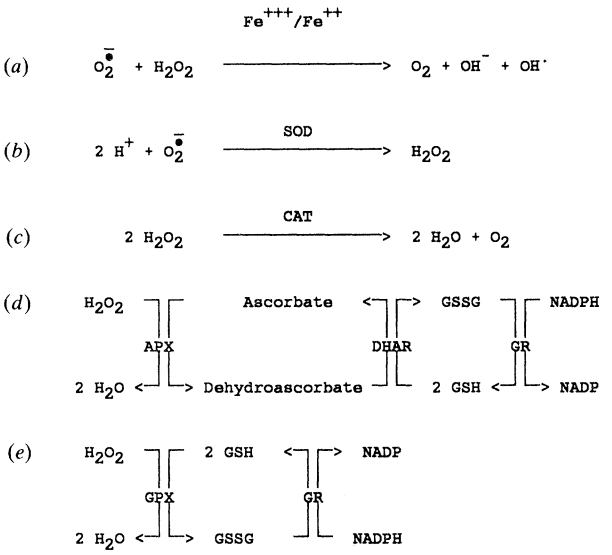

Figure 1. ROI-scavenging systems in eukaryotic cells. (*a*) Fenton reaction; (*b*) superoxide dismutase; (*c*) catalase; (*d*) ascorbate-glutathione cycle: ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR); (*e*) glutathione cycle: glutathione peroxidase (GPX), glutathione reductase (GR).

expression of the different genes coding for enzymes involved in the ROI-scavenging systems. In different species, numerous cDNAs coding for SODs (Bowler *et al.* 1993) and for enzymes involved in H_2O_2-scavenging systems (table 1) have been isolated. In the Laboratorium voor Genetica (Gent, Belgium), the expression pattern has been analysed of genes coding for ROI-scavenging enzymes in *Nicotiana plumbaginifolia* (SODs, CATs, GPx) and in *Arabidopsis thaliana* (SODs, APx). In *N. plumbaginifolia*, the different isoforms of SOD and CAT are encoded by separate nuclear genes and cDNA clones have been isolated for two mitochondrial MnSODs (Bowler *et al.* 1989; W. Van Camp, unpublished data), two chloroplastic FeSODs (Van Camp *et al.* 1990; W. Van Camp, unpublished results), a cytosolic Cu/ZnSOD (Tsang *et al.* 1991), a chloroplastic Cu/ZnSOD (D. Hérouart, unpublished data), and three catalases (H. Willekens, unpublished data). To evaluate the role of these different isoforms of SOD and CAT in protecting plants against oxidative stress, we determined their steady-state mRNA levels in leaves during environmental stress. It was shown that SODs are highly induced by adverse stress conditions, albeit not co-regulated (Tsang *et al.* 1991). For example, the cytosolic Cu/ZnSOD mRNA levels increased rapidly during heat shock, chilling, and after a paraquat treatment in the dark, whereas FeSOD mRNA levels were highly induced after a paraquat spray in the light (Tsang *et al.* 1991).

No significant variations of SODs, GPx, CAT2, and CAT3 mRNA levels in leaves were observed during a 12 h light/12 h dark photoperiod. Only the CAT1 mRNA level fluctuates during the normal photoperiod. After a prolonged maintenance in the dark (3 days), SODs and CAT1 mRNA levels dramatically decreased whereas elevated levels of CAT2 and CAT3 mRNA were detected.

Recently, we have started to analyse the effects of ozone on SOD gene expression using *Nicotiana tabacum* Petit Havana SR1. Plants grown in a 16 h photoperiod were fumigated with 100 p.p.b., 200 p.p.b., and 500 p.p.b. ozone for a 5 h period at the beginning of the normal photoperiod (see figure 2). Samples were taken immediately after fumigation and 2 h and 19 h afterwards. Cultivar SR1 is not particularly sensitive to ozone in comparison with tobacco varieties such as Bel W3 (Aycock 1982) and even a 5 h fumigation of 500 p.p.b. ozone produced only minimal damage which appeared two days after treatment. At the mRNA level, 100 p.p.b. and 200 p.p.b. doses produced no response (data not shown), but 500 p.p.b. had a very dramatic effect, as shown in figure 2*b*. Cytosolic Cu/ZnSOD mRNA levels increased approximately 12-fold during the period of ozone fumigation and the expression continued to increase for some time after treatment before returning to more normal levels. MnSOD mRNA levels only increased (about sevenfold) during the recovery period, and the elevated levels were maintained for at least 19 h. In contrast, FeSOD mRNA was almost undetectable after fumigation and only after a period of recovery did the transcript levels return to normal. Currently, we are analysing mRNA levels of ROI-scavenging enzymes in tobacco varieties more sensitive

Table 1. *Plant cDNAs coding for H_2O_2-scavenging enzymes*

enzyme	plant species	references
catalase	maize	Bethards *et al.* (1987); Redinbaugh *et al.* (1988)
	sweet potato	Sakajo *et al.* (1987)
	cotton	Ni *et al.* (1990)
	pea	Isin & Allen (1991)
	Arabidopsis	Chevalier *et al.* (1992)
	rice	Mori *et al.* (1992)
	tomato	Drory & Woodson (1992)
	N. plumbaginifolia	H. Willekens (unpublished)
ascorbate peroxidase	pea	Mittler & Zilinskas (1992)
	Arabidopsis	Kubo *et al.* (1992)
glutathione reductase	pea	Creissen *et al.* (1991)
glutathione peroxidase	*N. sylvestris*	Criqui *et al.* (1992)
	citrus	Holland *et al.* (1993)

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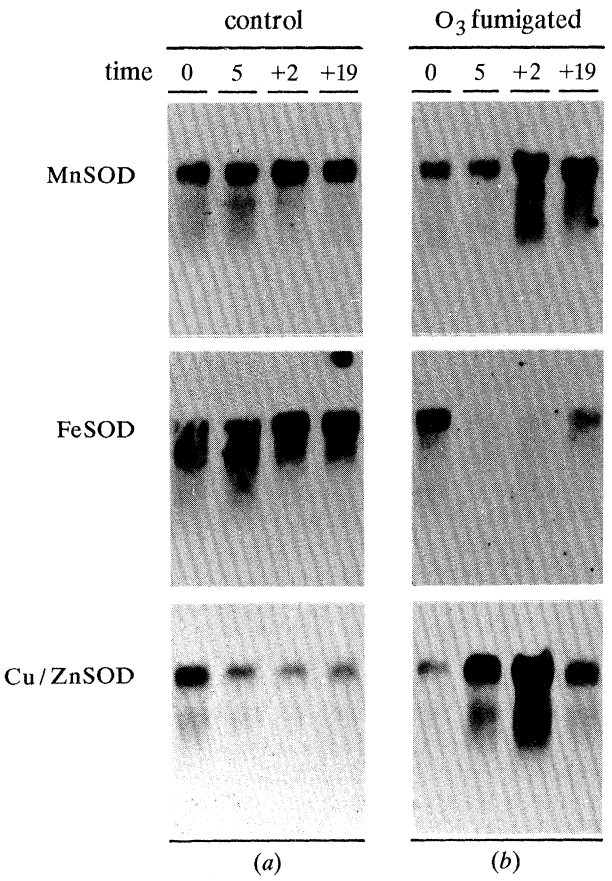

Figure 2. Effect of 500 p.p.b. ozone on SOD gene expression. Tobacco plants were kept in a 16 h photoperiod (light intensity 5000 lux) both before and during the experiment. Ozone was administered at the beginning of the normal photoperiod and was maintained at 500 p.p.b. for 5 h. Fully expanded leaves were taken for RNA analysis just before (time zero) and at the end of fumigation (time 5 h) and additionally 2 h (+2) and 19 h (+19) post-fumigation. Panel (*a*) represents the results from plants maintained in a growth chamber without ozone fumigation and panel (*b*) shows the results from ozone-fumigated plants. RNA isolation and hybridization were performed as described by Tsang *et al.* (1991).

to ozone. Preliminary results showed that CAT and GPx mRNA levels fluctuated considerably during ozone fumigation (120 p.p.b.) and post-fumigation.

To gain further insights into the molecular mechanisms of SOD induction, the promoter of the *N. plumbaginifolia* cytosolic Cu/ZnSOD gene has recently been isolated and fused to the β-glucuronidase reporter gene (*gus*) (Hérouart *et al.* 1993). Studies on Cu/ZnSOD reporter gene expression in protoplasts revealed that sulphydryl antioxidants such as reduced glutathione, cysteine, and dithiothreitol induce the expression of the cytosolic Cu/ZnSOD gene. In contrast, no induction of the chimeric gene was detected in protoplasts treated with the oxidized forms of glutathione and cysteine or with H_2O_2 and paraquat (Hérouart *et al.* 1993). Transgenic plants harbouring the cytosolic Cu/ZnSOD-GUS construct can also be used to monitor the cell-specific generation of superoxide radicals in the cytosol. For example, the SOD promoter directs the expression of *gus* gene specifically in the vascular tissues of leaves, in a punctate pattern indicative of phloem-specific expression. After a paraquat treatment, very strong GUS activity was also detected in photosynthetically active cells of leaves and in some epidermal root cells of seedlings (Hérouart *et al.* 1994).

3. ENGINEERING OXIDATIVE STRESS-TOLERANT PLANTS

Several studies suggest that a significant improvement in oxidative stress resistance requires overproduction of several enzymes. Elevated levels of SOD, APx, and glutathione reductase (GR) in *Conyza bonariensis* were correlated with resistance against paraquat (Shaaltiel & Gressel 1986) and photoinhibition (Jansen *et al.* 1989). In maize inbreds, resistance against paraquat and drought stress was correlated with high levels of both SOD and GR and not with high levels of only one of these (Malan *et al.* 1990). Paraquat-tolerant lines of perennial ryegrass showed significantly higher activities of both CAT and SOD as compared to susceptible cultivars (Harper & Harvey 1978). In paraquat-resistant callus of tobacco, CAT and APx activities are unaffected whereas SOD levels are increased two- to threefold compared to controls (Furusama *et al.* 1984).

Only recently, transgenic plants have been generated to evaluate the effect of overproduction of ROI-scavenging enzymes. Because superoxide radicals are commonly produced in illuminated chloroplasts, most of the experiments describe the effects of SOD overproduction in this cellular compartment (table 2). Initial results of Tepperman & Dunsmuir (1990) were discouraging. These authors showed that transgenic tobacco which overproduced a petunia chloroplastic Cu/ZnSOD is not more resistant to paraquat and to ozone as compared to non-transformed control plants (Pitcher *et al.* 1991). Moreover, transgenic tomato plants with two- to fourfold increases in chloroplastic Cu/ZnSOD do not exhibit tolerance to photoinhibitory conditions (Tepperman & Dunsmuir 1990). In contrast, recent results have shown that overproduction of pea chloroplastic Cu/ZnSOD in tobacco leaves can improve the tolerance to photoinhibition and to paraquat (Sen Gupta *et al.* 1993). As pointed out by these authors, the apparent discrepancies between their results and that of others can be largely explained by differences in assay methods and in levels of engineered Cu/ZnSOD gene expression. As such, it is thought that a paraquat treatment of transgenic plants which produce high levels of Cu/ZnSOD, creates a burst of H_2O_2 in chloroplasts, necessitating a very efficient H_2O_2-scavenging system. Moreover, Cu/ZnSODs are sensitive to end-product (H_2O_2) inhibition and an excess of H_2O_2 can simply inactivate the enzyme. Recently, increased tolerance to paraquat has been observed in several transgenic potato lines overproducing the cytosolic or the chloroplastic Cu/ZnSOD of tomato (Perl *et al.* 1993).

To avoid the problem of possible inactivation of the engineered Cu/ZnSOD by an excess of H_2O_2, we overproduced a *N. plumbaginifolia* H_2O_2-insensitive

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Table 2. *Oxidative stress tolerance of transgenic plants with elevated ROI-scavenging enzymes*

plant	engineered enzyme/origin	estimated increase of enzyme activity (fold)	tolerance	reference
tobacco	$Cu/ZnSOD_{chl}$ of pea	2	paraquat and photoinhibition	Sen Gupta *et al.* (1993)
	$Cu/ZnSOD_{chl}$ of petunia	30–50	no tolerance to paraquat	Tepperman & Dunsmuir (1990)
	$Cu/ZnSOD_{chl}$ of petunia	15	no tolerance to ozone	Pitcher *et al.* (1991)
	$MnSOD_{mit}$ of *N. plumbaginifolia* (targeted to chloroplast)	1.6–3	paraquat and ozone	Bowler *et al.* (1991) W. Van Camp (unpublished)
	$MnSOD_{mit}$ of *N. plumbaginifolia*	30	little paraquat tolerance	Bowler *et al.* (1991)
	GR of *E. coli* in cytosol	1–3.5	paraquat, no tolerance to O_3	Aono *et al.* (1991)
	GR of *E. coli* in chloroplast	3	paraquat and sulphur dioxide	Aono *et al.* (1993)
tomato	$Cu/ZnSOD_{chl}$ of petunia	2–4	no tolerance to chilling	Tepperman & Dunsmuir (1990)
potato	$Cu/ZnSOD_{chl}$ of tomato	not estimated	paraquat	Perl *et al.* (1993)
	$Cu/ZnSOD_{cyt}$ of tomato	not estimated	paraquat	Perl *et al.* (1993)

MnSOD in chloroplasts of tobacco (Bowler *et al.* 1991). A significant improvement in the protection against a paraquat treatment in light was observed in transgenic plants and particularly in old leaves (Bowler *et al.* 1991). Recently, these transgenic lines have been tested for ozone tolerance. Transgenic tobacco displayed approximately fourfold less ozone injury than a control line, when exposed to ambient levels of ozone (120 p.p.b. during the day) for 8 days (Van Camp *et al.* 1994). In contrast, mitochondrial overproduction of MnSOD in tobacco conferred enhanced tolerance to a paraquat treatment in the dark and not in the light, confirming that superoxide radicals are generated almost exclusively in chloroplasts during paraquat treatment in the light (Bowler *et al.* 1991).

To clarify the significance of glutathione reductase (GR) in plant systems for the detoxification of ROI, Aono *et al.* (1991) generated transgenic tobacco that contained 1–3.5 times higher GR activity than non-transformed plants by expressing the GR gene from *Escherichia coli* in the cytosol. These transgenic plants exhibited lower susceptibility to paraquat but they were no more resistant to ozone than were control plants. Recently, the same authors have shown that transgenic tobacco plants with high levels of *E. coli* GR in their chloroplasts exhibited lower susceptibility both to paraquat and to sulphur dioxide as compared to non-transformed control plants (Aono *et al.* 1993).

4. CONCLUSIONS AND PERSPECTIVES

In some cases, transgenic plants with increased levels of SOD exhibit substantial improvements in oxidative stress tolerance under laboratory conditions. However, it seems that only moderate increases of SOD provide such tolerance.

The effects of SOD overproduction are also controversial in mammals and *Drosophila*. In mammalian cells, increases of SOD up to fivefold can be compensated by comparable inductions in H_2O_2 scavengers such as GPx indicating that cells adapt themselves in favour of a constant $O_2^{\cdot-}/H_2O_2$ ratio (Ceballos *et al.* 1988; Kelner & Bagnell 1990). Analysis of our transgenic plants overproducing MnSOD in the chloroplasts showed no increase of APx, dehydroascorbate reductase (DHAR), or GR compared with non-transformed plants (Slooten *et al.* 1993).

The introduction of a H_2O_2-scavenging enzyme in parallel with SOD in plants might reduce the risk of imbalancing the $O_2^{\cdot-}/H_2O_2$ ratio. The choice between peroxidases and catalases is not simple. Peroxidase activities require reduced substrates and, hence, efficient endogenous mechanisms for the regeneration of these substrates. APx or GPx seem to be the enzymes of choice because they are associated with SOD in natural ROI-scavenging systems in eukaryotic cells. Alternatively, catalases which do not require other substrates than H_2O_2 for activity could be targeted to different cell compartments. However, catalases convert H_2O_2 to oxygen which can be converted back to $O_2^{\cdot-}$. Further molecular analyses of stress-responsive genes may help us to decide which H_2O_2-scavenging enzymes should be preferentially overproduced in combination with SOD.

Another approach to obtaining tolerance to oxidative stress without risk of imbalance of the $O_2^{\cdot-}/H_2O_2$ ratio may be the isolation and engineering of regulatory genes that control the synthesis of different enzymes involved in oxidative stress response. Jansen *et al.* (1989) showed that the levels of SOD, APx, and GR were higher in paraquat-resistant than in sensitive biotypes of *Conyza*. Moreover, activities of these three scavenging enzymes cosegregated with resistance in both the F1 and F2 generations. These results suggest that a dominant nuclear gene controls resistance by pleiotropically controlling the levels of these three enzymes. The isolation of such regulatory genes will be very important for the engineering of transgenic oxidative stress-tolerant plants.

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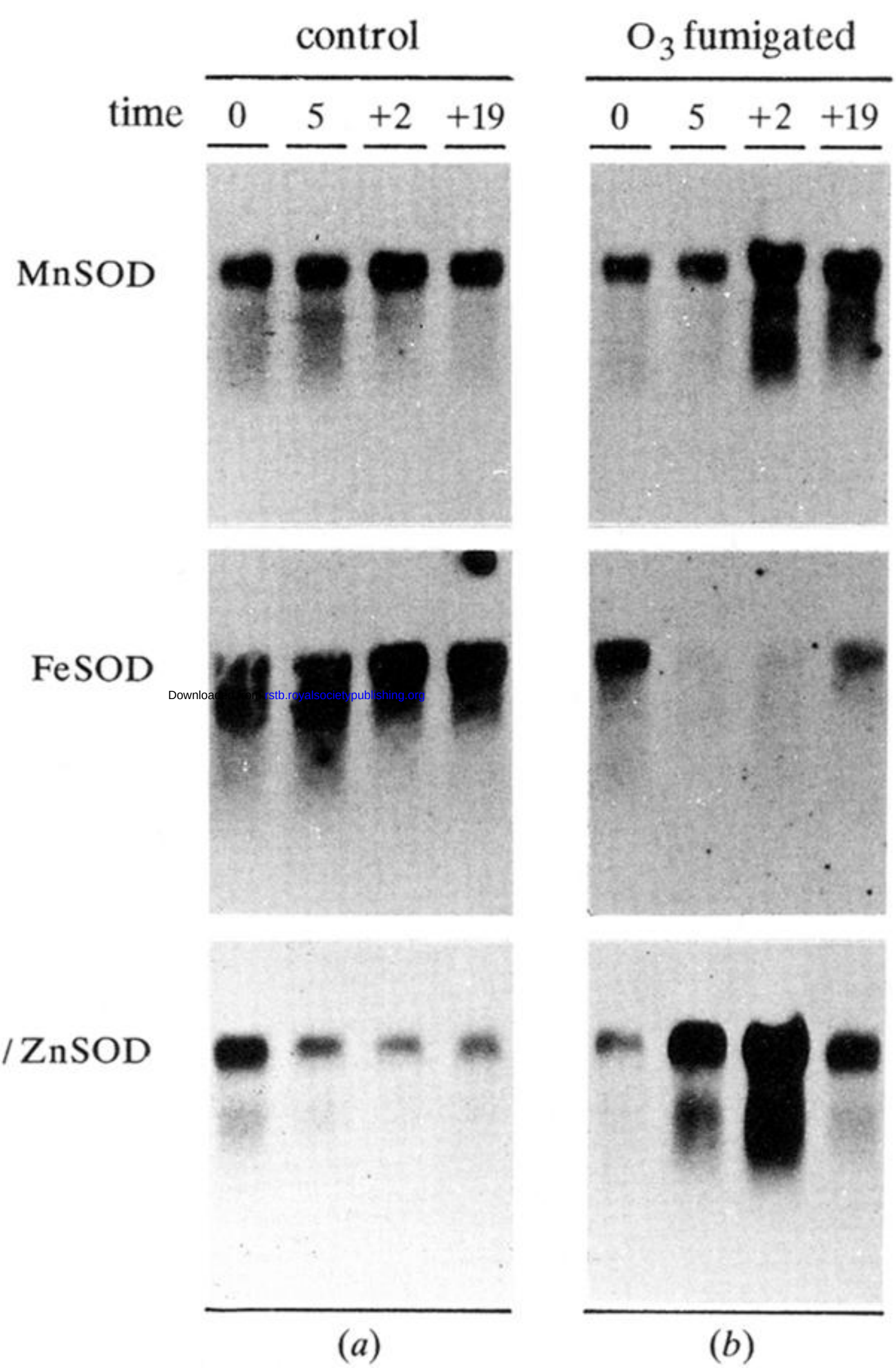

igure 2. Effect of 500 p.p.b. ozone on SOD gene ex-ression. Tobacco plants were kept in a 16 h photoperiod ight intensity 5000 lux) both before and during the xperiment. Ozone was administered at the beginning of the ormal photoperiod and was maintained at 500 p.p.b. for h. Fully expanded leaves were taken for RNA analysis just efore (time zero) and at the end of fumigation (time 5 h) nd additionally 2 h (+2) and 19 h (+19) post-fumigation. anel (*a*) represents the results from plants maintained in a rowth chamber without ozone fumigation and panel (*b*) ows the results from ozone-fumigated plants. RNA isola-on and hybridization were performed as described by sang *et al.* (1991).