Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 3108–3112, April 1993
Plant Biology

Redox-activated expression of the cytosolic copper/zinc superoxide dismutase gene in *Nicotiana*

(thiol molecules/transcription regulation)

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Contributed by Marc Van Montagu, December 24, 1992

ABSTRACT **Superoxide dismutases (SODs; superoxide: superoxide oxidoreductase, EC 1.15.1.1) play a key role in protection against oxygen radicals, and SOD gene expression is highly induced during environmental stress. To determine the conditions of SOD induction, the promoter of the cytosolic copper/zinc SOD ($Cu/ZnSOD_{cyt}$) gene was isolated in *Nicotiana plumbaginifolia* and fused to the β-glucuronidase reporter gene. Oxidative stress is likely to alter the cellular redox in favor of the oxidized status. Surprisingly, the expression of the $Cu/ZnSOD_{cyt}$ gene is induced by sulfhydryl antioxidants such as reduced glutathione, cysteine, and dithiothreitol, whereas the oxidized forms of glutathione and cysteine have no effect. It is therefore possible that reduced glutathione directly acts as an antioxidant and simultaneously activates the $Cu/ZnSOD_{cyt}$ gene during oxidative stress.**

As a side reaction to their normal oxygen consumption, all aerobic organisms produce reactive oxygen species such as superoxide radicals ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2), which can lead to DNA, protein, and membrane damage (1). Plants have to protect themselves against these oxygen radicals and possess a variety of enzymatic and nonenzymatic antioxidant mechanisms to prevent oxidation of cellular components (2, 3).

During oxidative stress, the balance between the scavenging capacity of antioxidant systems and the production of reactive oxygen forms is lost. The generated $O_2^{-\cdot}$ can be converted into H_2O_2 and O_2 by several isoenzymes of superoxide dismutase (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1). In *Nicotiana plumbaginifolia*, the different isoforms are encoded by separate nuclear genes and cDNA clones have been isolated for a mitochondrial MnSOD (4), a chloroplastic FeSOD (5), a cytosolic copper/zinc SOD ($Cu/ZnSOD_{cyt}$) (6), and a chloroplastic Cu/ZnSOD (D.H., unpublished data). The significance of the different isoforms of SOD has been investigated by biochemical and molecular approaches and it was shown that SODs are differentially regulated during environmental stress (for review, see ref. 7).

The variations of SOD expression during environmental stress presumably reflect the diversity not only of the oxidative mechanisms that produce the reactive oxygen species but also the mechanisms by which cells receive and respond to oxidative stress. Little is known about how the environmental signal is transmitted to a transcriptional regulator in plants during oxidative stress. In bacteria, three regulons are found to be activated by oxidative stress: the oxyR regulon inducible by H_2O_2, the soxR regulon inducible by superoxide-generating agents, and the soxQ regulon (for review, see ref. 8). In yeast, the expression of Cu/ZnSOD is transcriptionally regulated by copper (9). A treatment of soybean roots with copper also results in an increase of the $Cu/ZnSOD_{cyt}$ (10). In mammals, active oxygen species are involved in the regulation of transcription factors like NF-κB (11).

Here, we report on the identification of molecules that modulate the expression of the $Cu/ZnSOD_{cyt}$ gene by using as reporter a chimeric gene containing the promoter of the *N. plumbaginifolia* $Cu/ZnSOD_{cyt}$ gene fused to the coding sequence of β-glucuronidase (GUS).

MATERIALS AND METHODS

Cu/ZnSOD Gene Isolation. A *N. plumbaginifolia* genomic library, constructed in λ Charon 35, was screened with a ^{32}P-labeled $Cu/ZnSOD_{cyt}$ cDNA pSOD3 (6) as described (12). Phage DNA of five positive recombinant clones was digested with *Hin*dIII. Two *Hin*dIII fragments (3.7 and 3.4 kb) hybridized with the full-length $Cu/ZnSOD_{cyt}$ cDNA. Only the 3.4-kb *Hin*dIII fragment hybridized with a 5′ end cDNA probe (257-bp *Sau*3A fragment) and was subcloned into *Hin*dIII-cut pGem2. A *Hin*dIII/*Hin*cII fragment (2.6 kb) was subsequently subcloned into *Hin*dIII/*Hin*cII-cut pGem2 to produce pCZ12SOD that was sequenced on both strands by dideoxynucleotide chain termination (13).‡ Primer extension was carried out as described (14).

Promoter–GUS Constructions. Three fusions at the ATG start codon of the GUS reporter gene (noted pGUSSOD31, pGUSSOD32, and pGUSSOD33) were obtained after site-directed mutagenesis by PCR. To create pGUSSOD31, a 2.5-kb fragment was amplified by PCR using pCZ12SOD DNA as template, the mutated antisense Cu/ZnSOD*Nco*I primer (5′-GGCAACGGCCTTCACCATGGTGGTATGTGATC-3′) to create a *Nco* I restriction site at the ATG start codon, and the T7 primer as the second primer. The *Hin*dIII/*Nco* I-cut PCR fragment (2491 bp) was ligated into *Hin*dIII/*Nco* I-digested pGUS1 (15). Two PCR amplifications were performed to construct pGUSSOD32. The first PCR amplification was carried out with pCZ12SOD DNA as template, the T7 primer, and the mutated antisense Cu/ZnSOD*Bam*HI oligonucleotide A (5′-AAGAGAGCAAGAGAGGGATCCAATATGTCTG-3′) to create a *Bam*HI restriction site. The *Hin*dIII/*Bam*HI-cut PCR fragment (824 bp) was purified. The leader sequence of the $Cu/ZnSOD_{cyt}$ without intron was amplified by the second PCR using a mutated sense Cu/ZnSOD*Bam*HI oligonucleotide B, the mutated antisense Cu/ZnSOD*Nco*I primer, and the $Cu/ZnSOD_{cyt}$ cDNA pSOD3 as template. The *Bam*HI/*Nco* I-cut PCR fragment (64 bp) was purified. pGUSSOD32 was constructed by cloning the *Hin*dIII/*Bam*HI and *Bam*HI/*Nco* I PCR fragments into *Hin*dIII/*Nco* I-digested pGUS1. A sense 20-nucleotide primer (nucleotides 122–141) and the antisense Cu/

Abbreviations: SOD, superoxide dismutase; GSH, reduced glutathione; GUS, β-glucuronidase; MU, methylumbelliferone; $Cu/ZnSOD_{cyt}$, cytosolic copper/zinc SOD; DEDC, diethyldithiocarbamate; DTT, dithiothreitol; BSO, buthionine sulfoximine.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. L08253).

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ZnSOD*Nco*I primer were used to amplify the leader sequence including the first intron from the pCZ12SOD. The *Nco* I-cut PCR fragment (1637 bp) was ligated into *Hin*dIII (T4 polymerase filled-in)/*Nco* I-digested pGUS1 to create pGUSSOD33. Two independent clones for each construct were sequenced and tested for transient expression of GUS as described (16). pGUSSOD31 and pGUSSOD32 plasmids were excised as a *Pvu* II fragment and ligated into the *Sma* I-digested binary vector pGSV4 to produce pGSCZSOD31 and pGSCZSOD32, respectively. The vector pGSV4 (a gift of Plant Genetic Systems, Ghent, Belgium) contains the T-DNA border sequences, the spectinomycin/streptomycin-resistance gene, and the neomycin phosphotransferase gene (*nptII*) driven by the nopaline synthase promoter for selection of transformed plants. PCRs were carried out essentially as described (17) except that 10 ng of DNA was used as template.

Transformation of Tobacco. *Nicotiana tabacum* SR1 was transformed by the leaf disc assay as described (18). Kanamycin-resistant plants were tested for *in situ* GUS staining in leaf discs (19). Positive transgenic plants were transferred to soil in the greenhouse and self-fertilized. To determine the segregation ratio, 50 seeds for each line were germinated at 24°C on solid MS medium (20) containing 100 μg of kanamycin per ml in a cycle of 16 h light/8 h dark.

Isolation and Treatment of Protoplasts. Surface-sterilized leaves of mature plants were used for protoplast isolation as described (21). Protoplasts were adjusted to 5×10^6 per ml in 0.4 M mannitol solution (pH 7); 240 μl of protoplast solutions were distributed in wells of a microtiter plate containing different concentrations of chemicals to be tested (10 μl) and kept in darkness at room temperature. After overnight treatment, protoplasts were centrifuged at $70 \times g$ for 10 min and 200 μl of the supernatant was discarded. Protoplasts were washed twice with 0.4 M mannitol solution. Finally, 50 μl of a 2-fold concentrated GUS buffer (19) was added in each well, mixed vigorously with the concentrated protoplast solution (50 μl), and centrifuged at $200 \times g$. The supernatant was used for protein and GUS analysis. Each treatment was performed on three independently isolated protoplast preparations.

Protein was assayed by the Bradford procedure (22). Quantitative kinetic analysis of GUS activities was carried out by fluorometric assay as described (23). GUS activities in crude protoplast or leaf extracts (7 μg of total protein) were determined twice at 37°C and expressed as pmol of methylumbelliferone (MU) per min per mg of protein.

RESULTS

Cloning and Characterization of the Cu/ZnSOD$_{cyt}$ Gene. A genomic library of *N. plumbaginifolia* was screened using the Cu/ZnSOD$_{cyt}$ cDNA (6) as a hybridization probe. Five different genomic clones were isolated and characterized by restriction mapping and DNA gel blot analysis. The promoter was found to reside on a 2.8-kb *Hin*dIII/*Hin*cII fragment (see *Materials and Methods*). This fragment was ligated into pGem2 to create pCZ12SOD and subsequently sequenced. A comparison of the genomic sequence and the cDNA sequence revealed that the Cu/ZnSOD$_{cyt}$ gene contains a 1584-bp intron in the 5′-untranslated leader sequence (Fig. 1). Primer-extension analyses using an antisense 31-nucleotide primer overlapping the ATG start codon (nucleotides 1637–1667) and an antisense 31-nucleotide primer from the 5′ leader sequence upstream from the intron (nucleotides 90–120) demonstrated the presence of a single transcription initiation site (data not shown). DNA gel blot hybridizations were carried out to determine the copy number of the Cu/ZnSOD$_{cyt}$ gene in the *N. plumbaginifolia* genome (Fig. 2). Under low-stringency conditions, two major hybridization bands (3.7 and 3.4 kb) are seen in the *Hin*dIII-digested DNA, corresponding to the *Hin*dIII fragments present in the genomic clone. A weakly hybridizing *Hin*dIII fragment (6.5

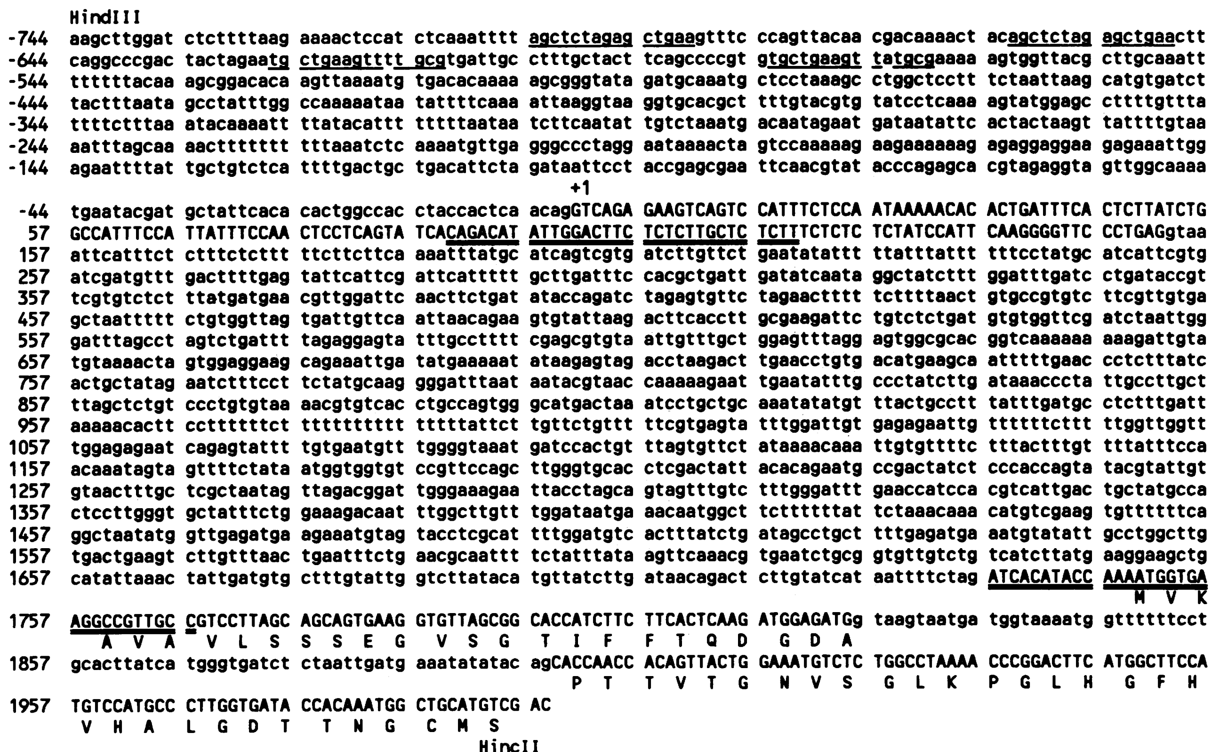

FIG. 1. Nucleotide sequence of the 5′ flanking region of the Cu/ZnSOD$_{cyt}$ gene. Nucleotides are numbered with the cap site designated +1. Capital letters indicate exon sequences. Lowercase letters represent introns and 5′ nontranscribed sequences. Double underlined sequences correspond to the sequence complementary to the two oligonucleotides used for primer extensions and PCRs. Underlined sequences correspond to direct repeated sequences.

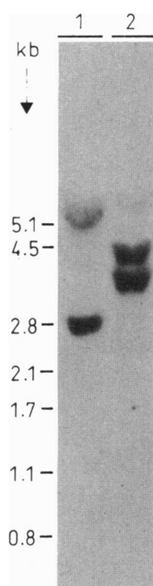

FIG. 2. DNA gel blot analysis of genomic DNA of *N. plumbaginifolia*. Genomic DNA (5 μg) isolated from leaves was digested with appropriate restriction enzymes (lane 1, *Eco*RI; lane 2, *Hin*dIII), separated by agarose gel (0.8%) electrophoresis, transferred onto a nylon filter, and then hybridized with ^{32}P-labeled Cu/ZnSOD$_{cyt}$ cDNA as probe.

kb) corresponds to the chloroplastic Cu/ZnSOD (J. Kurepa, personal communication). The 2.8-kb *Eco*RI fragment contains the entire coding sequence of the Cu/ZnSOD$_{cyt}$ and the 6.5-kb fragment hybridizes only to the 3′ end. Taken together, the DNA gel blot hybridizations suggest that Cu/ZnSOD$_{cyt}$ is encoded by a unique nuclear gene.

Analysis of Cu/ZnSOD$_{cyt}$–GUS Chimeric Genes. Three different DNA sequences were transcriptionally fused to the ATG start codon of GUS by PCR approaches. pGUSSOD31 contains the 5′ flanking region upstream of the ATG codon, pGUSSOD32 is identical to pGUSSOD31 except that the first intron is deleted, and pGUSSOD33 contains only the 5′ leader sequence including the first intron. Similar GUS activities were measured in protein extracts of protoplasts electroporated with pGUSSOD31 and pGUSSOD32, whereas no activity was detected with pGUSSOD33 (data not shown). Hence, the intron alone has no promoter activity and the presence of the intron in the leader sequence appears to have no effect on the transient expression levels of the promoter–GUS chimeric gene. Only the pGUSSOD31 and pGUSSOD32 constructions were cloned in a binary vector and introduced into *N. tabacum* via *Agrobacterium*. Of 30 kanamycin-resistant Cu/ZnSOD–GUS-transformed plants (15 for each construction), 16 showed GUS activity in leaf discs. After self-fertilization, 6 independent plants containing one functional T-DNA locus (data not shown) were selected for further studies. The analysis of GUS activity in leaves of the different lines (Table 1) showed that the presence of the intron in the leader sequence has no apparent influence on the expression of the chimeric genes, confirming our results obtained by transient expression in protoplasts.

Activation of the Cu/ZnSOD$_{cyt}$ Promoter in Transgenic Protoplasts. The aim of this study was to identify molecules that affect the expression of the Cu/ZnSOD$_{cyt}$ gene. To test many compounds in various conditions, a protoplast system in microtiter plates was developed (see *Materials and Methods*). The initial analysis was performed with the transgenic lines that contained the pGSCZSOD31 T-DNA (CZGUS311, CZGUS313, and CZGUS315).

Table 1. Determination of GUS activities in leaves of different transgenic plants by fluorometric assay

Plant lines	T-DNA plasmid	GUS activity, pmol of MU per min per mg of protein
CZGUS311	pGSCZSOD31	4428.8 ± 597.9
CZGUS313	pGSCZSOD31	4444.4 ± 691.7
CZGUS315	pGSCZSOD31	4287.4 ± 559.0
CZGUS321	pGSCZSOD32	3140.1 ± 418.3
CZGUS322	pGSCZSOD32	4911.4 ± 457.2
CZGUS326	pGSCZSOD32	3502.4 ± 368.9

pGSCZSOD31 contains the 5′ flanking region upstream of the ATG codon and pGSCZSOD32 is identical to pGSCZSOD31 except that the first intron is deleted. Pieces (2 cm^2) of the third and the sixth leaves counted from the apex from each plant were used for protein extraction, and GUS activities in protein extracts (7 μg) were determined twice.

To explore the possibility that copper can influence the transcription of the Cu/ZnSOD$_{cyt}$ gene, transgenic protoplasts were incubated overnight in solutions containing different concentrations of $CuSO_4$. Concentrations of $CuSO_4$ ranging from 1 μM to 10 mM did not affect GUS activity, whereas a higher concentration (100 mM) was found to be toxic. The effect of a depletion of copper was tested by using different divalent cation chelators such as diethyldithiocarbamate (DEDC), dipicolinic acid, and EDTA (Fig. 3*A*). Again no effect was detected except for DEDC, where a concentration-dependent increase of GUS activity was measured (Fig. 3*A*). DEDC is a thiol drug and we tested the effect of several other sulfhydryl molecules on the expression of the chimeric Cu/ZnSOD–GUS gene. Activation of the Cu/ZnSOD$_{cyt}$ promoter was also found in transgenic protoplasts after incubation with reduced glutathione (GSH), L-cysteine (Cys), and dithiothreitol (DTT) (Fig. 3*B*). The highest increase of GUS activity was measured after a treatment with 10 mM GSH and 10 mM DTT, whereas higher concentrations of L-cysteine (100 mM) were necessary to induce the same level of GUS activity. No increase of GUS activity was observed with oxidized glutathione and cystine (Fig. 3*B*). Expression of the chimeric gene was also induced after a treatment with L-cysteine and *N*-acetyl-L-cysteine, whereas no effect was observed with two other amino acids, methionine and serine, which are structurally identical to cysteine except for the absence of the sulfhydryl group (Fig. 3*C*). Hence, except for 2-mercaptoethanol, all thiol molecules tested induce GUS expression driven by the Cu/ZnSOD$_{cyt}$ promoter.

Because cysteine is a precursor for GSH synthesis (for review, see ref. 24), the observed induction by cysteine can be either direct or indirect by enhancing GSH biosynthesis. L-Cysteine can induce the expression of the chimeric gene, whereas treatment with glutamate and glycine, the other precursors for GSH, did not affect GUS activity. Using protoplasts pretreated for 1 day with 10 mM buthionine sulfoximine (BSO), an inhibitor of the γ-glutamylcysteine synthetase (25), treatment with L-cysteine can still induce the expression of the chimeric gene, but the induction was reduced compared to the control (Fig. 4).

The induction of GUS expression driven by the Cu/ZnSOD$_{cyt}$ promoter with L-cysteine (10 mM) was detectable 4 hr after the beginning of treatment and reached a maximum after 6 hr (data not shown). During the first hour of treatment, both reduced and oxidized forms of glutathione generated a slight decrease in GUS activity in treated protoplasts. A significant increase in GUS activity was measured after 7 hr in protoplasts that were incubated with GSH (1 mM) (data not shown).

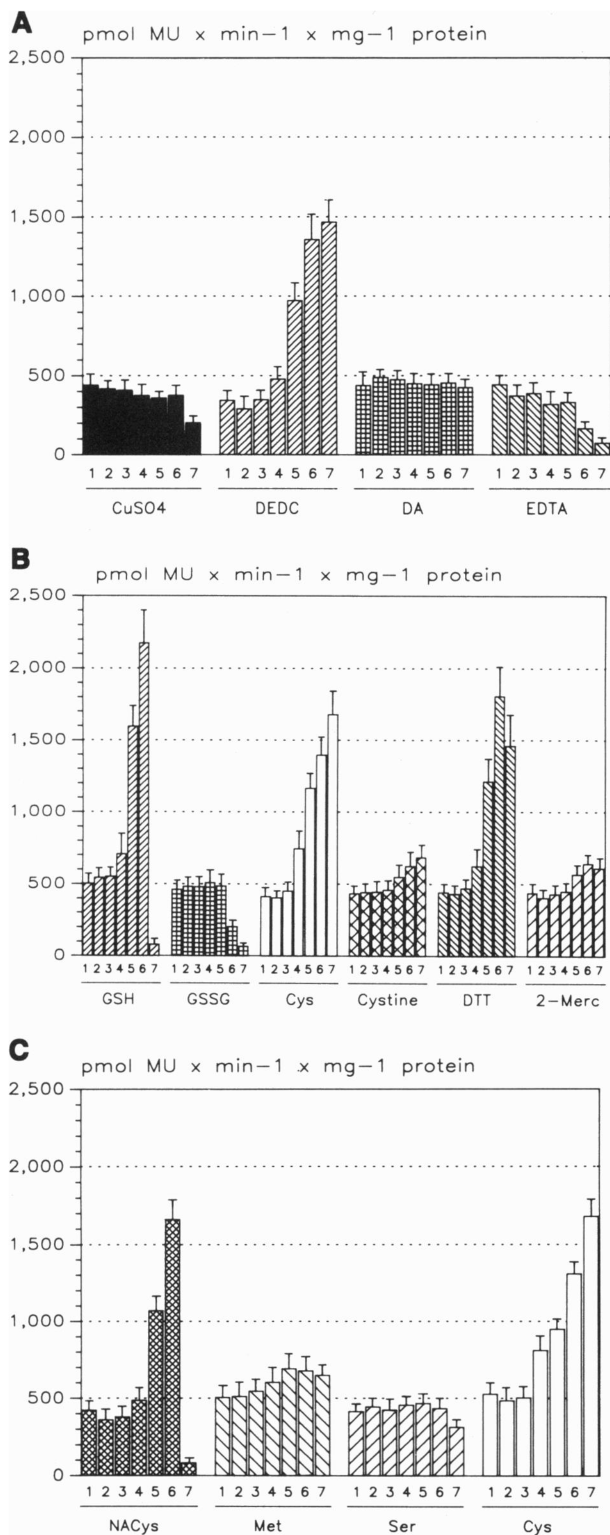

FIG. 3. Analysis of GUS activity in transgenic protoplasts after treatment with several components. Three independently isolated protoplast solutions were incubated overnight in darkness at room temperature in microtiter plate wells containing different concentrations of compounds (see text) (1, control; 2, 1 μM; 3, 10 μM; 4, 100 μM; 5, 1 mM; 6, 10 mM; 7, 100 mM). GUS activities were determined twice at 37°C. DA, dipicolinic acid; GSSG, oxidized glutathione; NACys, *N*-acetyl-L-cysteine.

Induction of GUS chimeric gene expression in protoplasts by GSH and L-cysteine is specific to the promoter of Cu/ZnSOD$_{cyt}$, since no induction was detected in protoplasts

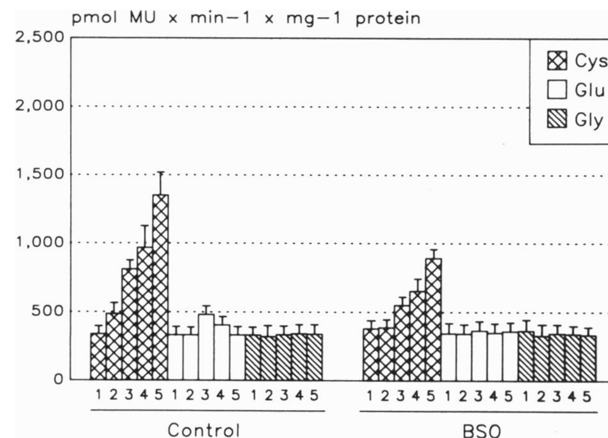

FIG. 4. Effect of pretreatment of protoplasts with BSO on induction of chimeric gene expression by L-cysteine. Preincubations of protoplasts were performed during 1 day in 0.4 M mannitol solution (control) or in solution containing 0.4 M mannitol and 10 mM BSO. Pretreated protoplasts were washed twice with 0.4 M mannitol solution and then incubated overnight in the presence of different concentrations of L-cysteine, glutamate, and glycine (1, control; 2, 10 μM; 3, 100 μM; 4, 1 mM; 5, 10 mM).

prepared from transgenic plants that have integrated a Mn-SOD/promoter–GUS gene (MnGUS11; kind gift of W. Van Camp) or an extensin/promoter–GUS gene (ExtGUS6; kind gift of C. Tiré) (Fig. 5). Moreover, the increase in expression of the chimeric gene by GSH, L-cysteine, and DTT was observed in protoplasts that were prepared from CZGUS321, CZGUS322, and CZGUS326 transgenic lines, showing that the presence of the first intron in the leader sequence has no influence on induction of the Cu/ZnSOD$_{cyt}$ promoter by thiol molecules (data not shown).

DISCUSSION

To study the effect of redox alterations on transcription of the Cu/ZnSOD$_{cyt}$ gene in *N. plumbaginifolia*, we have characterized the 5′ flanking region of the Cu/ZnSOD$_{cyt}$ gene. As in the rice Cu/ZnSOD$_{cyt}$ gene (26), the nontranslated region of the gene contains an intron, but the presence of this intron does not modulate the quantity of GUS activity driven by the *N. plumbaginifolia* Cu/ZnSOD$_{cyt}$ promoter in transgenic protoplasts.

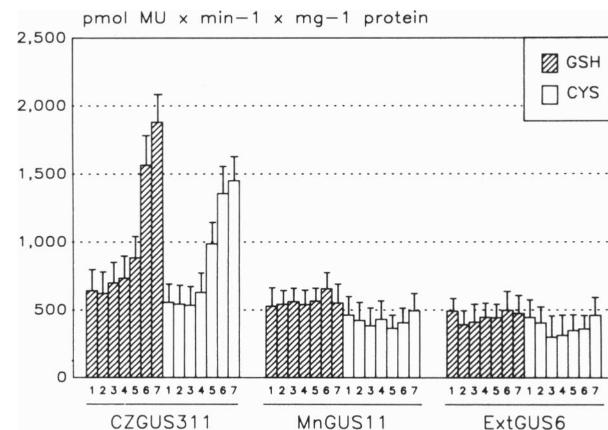

FIG. 5. Analysis of induction of GUS activity in protoplasts prepared from different transgenic tobacco lines. Incubations in thiol solutions were performed as described in Fig. 4 with different concentrations (1, control; 2, 100 nM; 3, 1 μM; 4, 10 μM; 5, 100 μM; 6, 1 mM; 7, 10 mM).

An oxidative stress situation has been defined as an alteration of the steady-state concentrations of components of cellular redox systems in favor of the oxidized form (27). Paradoxically, antioxidant sulfhydryl reagents like GSH, cysteine, or DTT cause a marked induction of Cu/ZnSOD$_{cyt}$ gene expression, whereas the effect is lost with oxidized forms of glutathione and cysteine. Moreover, no induction of the chimeric gene was detected in protoplasts treated with H_2O_2 and with the superoxide-generating herbicide paraquat (data not shown).

The most abundant nonprotein thiol molecule in plant cells is glutathione, which is present at millimolar concentrations (28). Glutathione appears to play a key role in protection against oxygen radicals (29). The level of GSH in foliar tissues has been shown to increase under various oxidative stress conditions, such as exposure to ozone, sulfur dioxide, heat shock, or drought (29), and this increase was followed by enhanced SOD activity in pea during SO_2 exposure (30). Our results showed clearly that addition of GSH into the medium can activate, directly or indirectly, expression of the Cu/ZnSOD$_{cyt}$ gene in protoplasts. Extracellular GSH was also shown to act as an activator of the transcription of genes encoding the cell wall hydroxyproline-rich glycoproteins and the phenyl propanoid biosynthetic enzymes in suspension-cultured cells or protoplasts of bean, soybean, and alfalfa (31, 32). Glutathione is taken up by the plant cells and the transport mechanism has been described recently for cultured tobacco cells (33). Hence, the observed increase of GSH during oxidative stress can serve two functions. Glutathione can act directly as an antioxidant and simultaneously activate the panoply of stress genes, including the Cu/ZnSOD$_{cyt}$ gene.

The action mechanism of the thiols on plant protoplasts is probably not mediated by the production of $O_2^{-\cdot}$ via autoxidation of thiol compounds, since Misra (34) showed that autoxidation only occurs at high nonphysiological pH (pH > 9). Direct inhibition of Cu/ZnSOD$_{cyt}$ activity by thiol molecules could lead to oxidative stress. However, this possibility can be excluded because no direct effect of the thiols (up to 10 mM) was observed *in vitro* on the activity of the Cu/ZnSOD$_{cyt}$ on native gels (data not shown). Two different hypotheses can be postulated to explain the action mechanism of the thiols. First, the thiol molecules can reduce a disulfide bridge, thereby activating a protein involved in reception or signal transduction of the oxidative stress response. Cleavage of disulfides of the β-adrenergic receptors by thiol compounds appears to activate the receptor in a manner similar to agonist binding (35), and this mechanism was proposed for many other cell-surface receptors in animals (36). The second hypothesis is the direct or indirect activation of a transcription factor by thiols. In animal cells, an unusual posttranslational modification involving reduction–oxidation regulates *in vitro* the DNA-binding activity of the transcription factors Fos, Jun (37), and NF-κB (38). Reduction or oxidation could provide a general mechanism for posttranslational control of transcription factors functioning in a fashion analogous to phosphorylation. Hence, further analysis of the trans-acting factors that interact with the promoter region of the Cu/ZnSOD$_{cyt}$ gene may provide a key for the dissection of oxidative stress mechanisms that control the induction of plant SOD genes.

We thank Sergeï Kushnir and Wim Van Camp for valuable discussions; Chris Genetello, Jan Gielen, and Luc Van Wiemeersch for expert technical help; and Martine De Cock, Karel Spruyt, and Vera Vermaercke for help in preparing the manuscript. This research was supported by grants from the Services of the Prime Minister (Interuniversitaire Attractiepolen 120CU192), the "Vlaams Actieprogramma Biotechnologie," and the International Atomic Energy Agency (Grant 5285). D.H. is indebted to the Ministère de la Recherche et de l'Espace (France).

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