

The tomato gene for the chloroplastic Cu,Zn superoxide dismutase: regulation of expression imposed in transgenic tobacco plants by a short promoter

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Abstract

The chloroplastic Cu,Zn superoxide dismutase (SOD) has an important role in the defense against damage by oxygen radicals in the chloroplasts. Here, for the first time, we report on the isolation of a genomic DNA clone from tomato that contains all the coding sequence for the chloroplastic Cu,Zn SOD as well as a 442 bp DNA fragment upstream of the translational initiation site. The latter upstream sequence has a putative TATA box and a 285 bp promoter region, 5' of the apparent transcriptional initiation and a 157 bp leader region. The coding sequence is composed of 8 exons that are interspaced by 7 introns; we termed this gene *SODCp;Le:1*. The 442 bp fragment was cloned into a pBI101 vector, upstream of the *uidA* (GUS) gene, via transcriptional fusion. *Agrobacterium*-mediated transformation resulted in transgenic tobacco plants.

The progeny (after self-pollination) of 14 transformed plants, which expressed GUS above a threshold of 1 nmol/min per mg protein, were found to fall into two distinct groups. In the seedlings of 10 lines (group A) GUS expression was enhanced by exposure to light. In 4 lines of this group maintenance for 3 days in the dark eliminated GUS activity. The seedlings of group B expressed GUS regardless of the light/dark regime. In plants of group A, GUS expression was also developmentally regulated: high GUS activity in young leaves, low activity in mature leaves and no activity in the roots. The results suggest that this short chloroplastic Cu,Zn SOD promoter contains motifs for developmental (spatial) regulation as well as motifs responsive to light (or to oxygen radicals resulting from light-driven photosynthesis).

Introduction

The enzyme superoxide dismutase (SOD, 1.15.1.1) was first described 25 years ago by

McCord and Fridovich [19] in bovine erythrocytes. It catalyzes the conversion of superoxide (O_2^-) to O_2 and H_2O_2 . Since its discovery numerous studies have established SOD as a key

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X77175 and X77176.

enzyme and the first link in the cascade of enzymes that scavenge toxic oxygen radicals in animals, plants and microorganisms [see 2, 5, 9, 10, 23 for reviews]. SOD activity is manifested by several isoforms that differ in metal content and cellular compartment. Plants contain Cu,Zn SOD in the cytosol as well as Cu,Zn SOD and (at least in some plants) Fe SOD in the chloroplasts, while Mn SOD resides in their mitochondria. Each of these isoforms is encoded by a different nuclear gene. The amino acid sequences of several plant Cu,Zn SODs were first published in 1986 [14, 15, 28]. The full sequence of the cDNA coding for the chloroplastic Cu,Zn SOD was first reported by our laboratory [22] for the tomato gene, by Scioli and Zilinskas [27] for the pea gene, and by Teperman *et al.* [29] for the petunia gene. The chloroplastic Cu,Zn SOD-coding sequence probably exists as a single gene in the tomato genome on chromosome 11 [20, 22]. The deduced amino acid sequence of the mature protein in tomato, pea and petunia is very similar but the respective transit peptides differ considerably. The genomic sequence that codes for plant cytosolic Cu,Zn SOD was revealed [24]. We are not aware of previous information on a genomic sequence that encodes the chloroplastic Cu,Zn SOD of plants.

The availability of the cDNAs encoding the chloroplastic and the cytosolic Cu,Zn SODs enabled tracing the transcript levels from these two genes in tomato [21, 22]. We found that the expression of both genes is developmentally and spatially regulated and the transcript levels changed considerably after exposure to stress conditions; but the two genes displayed very different patterns of expression during plant organ development and upon exposure to stress. Similarly, Tsang *et al.* [31] found that the transcript levels of the genes coding for the cytosolic Cu,Zn SOD and the chloroplastic Fe SOD are differentially affected by different stress conditions. Acevedo and Scandalios [1] used the cDNAs of the cytosolic Cu,Zn SOD and the mitochondrial MnSOD to reveal changes in transcription of the respective genes in maize tissues. The activation of the SOD genes by the light-sensitized phyto-toxin cercosporin is noteworthy. While the tran-

script level was drastically increased by cercosporin, the level of the SOD polypeptide remained stable, suggesting an increase in the turn-over of the enzyme [32].

The photosynthetic machinery in the chloroplast is very sensitive to damage by toxic oxygen radicals that are generated by photosystem II. The level of these radicals is elevated under stress conditions imposed by extreme temperatures and very high radiation fluence. The chloroplastic Cu,Zn SOD has a central role in scavenging toxic oxygen radicals, thus the mode of the regulation of the expression of the gene that codes for this enzyme is an interesting subject.

Here we report on the isolation of the chloroplastic Cu,Zn SOD gene from a genomic library of tomato and on the utilization of a promoter sequence from this gene, fused to a reporter gene, in order to obtain transgenic tobacco plants harboring this chimeric transgene. The capability of this promoter sequence to regulate gene expression was then traced in different tissues and in response to environmental effectors.

Materials and methods

Isolation and sequencing of the chloroplast Cu,Zn SOD gene from a genomic library of tomato

A genomic library (Charon 4A) of tomato (*Lycopersicon esculentum*) constructed by partial *Eco* RI digest was obtained from Dr J. Truettmer (UCLA, Davis, CA). It was screened by standard procedures [3], using the cDNA [22] that codes for the chloroplastic Cu,Zn SOD of tomato as radioactive probe. The *Eco* RI 13.5 kb fragment that was isolated was first truncated to 7 kb and then digested by several restriction enzymes; the fragments were then cloned into pUC18. The fragments were sequenced by using either universal primers or synthetic oligonucleotides that were synthesized on the basis of respective relevant cDNA sequences. DNA sequencing was by the dideoxynucleotide chain-termination method of Sanger *et al.* [26] with due modifications [3]. Essential fragments (e.g. upstream of the translational initiation site) were verified by PCR

sequencing using an automatic sequencing machine (Applied Biosystem 373, DNA Sequencer).

Primer extension analysis

A 20 bp long oligonucleotide with the sequence 5'-GGTGAAGATTGAGTGGGCGG-3' complementary to positions 162 to 181 of the genomic clone was used to map the transcriptional initiation site of the chloroplastic Cu,Zn SOD gene. Primer extension was performed on tomato leaves total RNA (20 μ g per reaction). The annealing and the polymerization reaction with AMV reverse transcriptase (Pharmacia) was carried out as described by Sambrook *et al.* [25].

Construction of the promoter::uidA (GUS) chimeric gene and transformation of tobacco

For the isolation of a DNA fragment, consisting of 442 bp upstream of the translational initiation, the chloroplastic Cu,Zn SOD gene (inside of pUC18) was first digested with *Nco* I and the latter site was blunt-ended. A further digestion with *Xba* I (in the polylinker of the plasmid) released the required DNA fragment, CSP (chloroplastic Cu,Zn COD short promoter). This fragment (469 bp) was ligated into the *Sma* I/*Xba* I restriction site of the vector pBI101 [12] resulting in the transcriptional fused gene CSP::uidA (Fig. 1). The chimeric gene-containing vector was

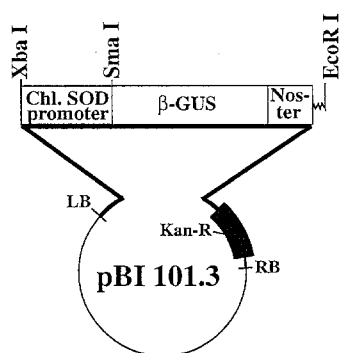


Fig. 1. Scheme of the engineered pBI101.3 vector, that was transferred into *Agrobacterium tumefaciens* LB 4404 cells. The chl. SOD promoter, termed in the text CSP (i.e. 442 upstream of the first codon) was cloned into the vector.

then introduced into *Agrobacterium tumefaciens* (LBA 4404) cells by electroporation and the transduced bacteria were selected on a medium containing kanamycin (25 μ g/ml) and streptomycin (300 μ g/ml). Plant transformation was performed basically as described by Horsch *et al.* [11]. Briefly, leaves from *in vitro* grown *Nicotiana tabacum* cv. Xanthi were infected with agrobacteria and incubated on shoot regeneration medium for one or two days. Thereafter the leaves were transferred to the same regeneration medium but plus 100 μ g/ml kanamycin and 400 μ g/ml carbenicillin. Shoots that regenerated were transferred to solidified Nitsch medium that also contained kanamycin (100 μ g/ml) and rooted plantlets were transferred to the greenhouse. Seeds from self-pollinated to plants were sterilized and germinated on solidified Nitsch medium containing kanamycin; the germinated seedlings were used as T₁ plants.

Detection of GUS activity

For the fluorometric assay tissues (0.5–1.0 g of leaves, roots or seedlings) were frozen in liquid nitrogen and ground in (1–2 ml) GUS extraction buffer (50 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 10 mM 2-mercaptoethanol). For seedling assays 7 or 8 samples were extracted. For greenhouse-grown plants, leaves or roots from two plants were sampled. The homogenate was centrifuged (10 000 $\times$ g, 20 min, at 4 °C) and aliquots of the cleared extracts were assayed fluorometrically using the substrate MUG (4-methylumbelliferyl β -D-glucuronide) as described [12]. GUS activity was normalized to protein concentration for each sample and calculated as pmol or nmol of 4-MU produced per minute per mg of soluble protein. Protein content was measured by the Bradford method [6] using BSA as a standard.

SOD activity gels

Samples (60–100 μ g protein per lane) from the above noted cleared extract were used for SOD

-285 GAATTCATAGTTCATTATTAAATTTAACTCTAAATACAAATATAGAGAAGACAAAGTTGATTGAGCACCAACAAACCAAAACAATGTT -186
 -195 TAAATAATTGTATATCCAAATTAATATTTTATTTACCCACCAACAACTTTTAACTTTTATGTTATACAACTAATATACCATTTTATATATATGCAA - 86
 - 85 AGTATGATAAAGCACCATTATGTGTTATTTTCCAAAAAATAATATATAGAAATATCATAAAGTAATTATTATGTCCAAATTTGTTAACCCA 14
 15 AAAATGAACATTAAAAATGTCAAATAGATCTTGCAAACCTGACAAATCTAAGAAAAACCCCATAAAACTCAAAGATAAAATCTTGTTCTACTGCTATC 114
 115 TAAACATTTATCATTTTATCAATTCATCATTGTGGGAGGAGCATGGCGGCCACTCAATCTTCACCACAACCAGCACTACCAATTTCTTCTTATACCCA 214
 M A A H S I F T T T S T T N S F L Y P
 215 ATTTCTTCTTCTTCTTCTTCCCTAACATTAACCTCTTCTTCTAGGTGTTTCACTTAATGTTAATGCAAAATTTGGCCAATTTTGACCTTTATGCTG 314
 I S S S S S S P N I N S S F L G V S L N V N A K F G Q S L T L Y A V
 315 TCCTACCCCTTAAACCCCTTACTGTTTTGCTGCTACTAAGAAAGCTGTGCTGCTCTTAAGGGCAACTCTAATGTTGAGGGGTTGCTACTGTATCTCA 414
 T T P K P L T V F A A T K K A V A V L K G N S N V E G V V T L S Q
 415 AGATGATGATGGGCCAATTTCTTTTATAGCTTTAATTGCTGCTTTGGGTTATGTATGTGGATTAAAGATGTAATCTTTATTTACTAATAGGTTCTATACC 514
 D D D G P T
 515 CTTTATCTGAATCTTTATCTGTTCAATTTCCACCATTTTGTGATGTAATTTGTTGTTTTAGATGTAATCTTTATTTGCTAATGGCTCATACCTTTGTT 614
 615 GAATTTCCACCCATTTTGTGCTTTATTTCTACTGATTTGTATCGTTTACTAGAGTGAGGCTATCTTAAACAGCCTCTCTGCCTTAAACAGGTATGGG 714
 715 TAAGTTGCGTACACACATCTATCCCTAGACCTCACTTGTGGGATTATGCTGGGTTGTTGTTGTTGCGATTGTGACAAATAGCTGAATCTTTATTTACTAA 814
 815 TGTTCATACCTTTGCATCTGAATCTTTATCTGTTTATTTTCCACCATTTTGTTTGTATTTTATTTATTTCTTGGATTGTGACCTTAAAGAGTAATCT 914
 915 TTTGTTTACAAATGTTATATTTCCCTTTATCTAAGCTCTATATGTTAATGTCACCTTATGCTCTATTTTGTGTTGTTGTCACATTTGTGTAGTGAAC 1014
 1015 TAAAGATGTCACCCCTTTCTAAATACAGGTTTACACCCCTTTTATCTGAATCTCTATCTGTTTGCATTTCCACCATTATGCTCTAATTTGCTCTTCAGTAT 1114
 1115 CACATTTGTGATTGCTAGCTAAAGATGTAAGTCTTCTGAGTATATTTGTAATGTGATTGTACCTAAAGATGTAATCTTTATTAAGAAGTCTTCTGATACC 1214
 1215 -CTTCTATCTGAATCTTTATCTGTTTGCATTTCCACTCCTTATGCACTCAATTTGCCTTTTCTTTTGTAGACTTGTGCACTATTGCTTTCTTGTGCA 1314
 1315 CACTTGGATGTGGATTCTGCTAAGTCTGAGCTTCTCTAGAGTATTTCTGAGCAATTTACTCCAAATCTTTATCTCTATTTCTTGTGCTAGTACTCATATGT 1414
 1415 CCTTATTTGGTTTGTATACAATATTTTGTGTTGGACCGAAAGATGTAAGCTTTATAGGAATATTTTACGCCCTTATGATTGAGTATTTTTTTCTTCTT 1514
 1515 TGTATAGGTCACACTACGGTTAATGTTCTGATAACTGGACTTGTCTCTGGACTTACCGGTTTTCATTTAGTAAGTTATGGTTGCTACTATTGAAATTATA 1614
 T V N V R I T G L A P G L H G F H L
 1615 CTGCTTCTGTATGACTTGTCTTGTGGCTAATTTATGGTAATTTTGTGTTAAATGTTATGCTTGGCTGTGTTGTAGCATGAATATGGTGACACTACA 1714
 H E Y G D T T
 1715 AACGGATGATGTCTCACAGGTTAGCTTCTGATTAGTTTGAACCTTTGAGTTAATCGAAATGTTGATGAATTGGAACCTCCTTGTAAATGTACTAAAATA 1814
 N G C M S
 1815 AAATTGGAATCTTCTAGTAGCTAGTTGGAATGATGTGTTAGTTCTAGTTTCTGCTGGTACTAGTTTGAATTTGTTGGGTTGATTACGACAGGAGCCCC 1914
 T G A H
 1915 ATTTTAAATCCGAATAAATGACACATCGAGCTCCTGGAGATGAAATCCGTCATGCGGGTACCTGGGAAACATAGTGGCAATGCCGATGGTAAAGTACT 2014
 F N P N K L T H G A P G D E I R H A G D L G N I V A N A D
 2015 ACTTTCTATCTTTCATAGCGCTTGTATCTGTATTTGTTGTTTGAAGTGGATGATGACTTTCTAGATTCTTAATTTGCGCTTACTGTATGACGGCTGGCT 2114
 G V A
 2115 GAAGTGACACTTGTAGATAATCAGATTGTACATTACCTTGTCTGGTCTAACGTTGCAATTCTAGTTCAACTTTATAGTCTCTCTAGTTTGTATTTGT 2214
 E V T L V D N Q
 2215 TATAGTTTATAGATAATTACTGACTCTAAATTTTGTGTTTGTATGTATAGTTTATAGATATTACTGACTCTAAATCTTGTGCACCAATCAGATACCATTGA 2314
 I P L T
 2315 CCGGTCCAACTCAGTTGTTGGACGAGCACTTGTGGTTCTAGGCTTGTAGGATGACCTCGGAAAGTAAGTTCTACATTTTGTATNTGATCCATCTAATC 2414
 G P N S V V G R A L V V H E L E D D L G K
 2415 TGTAAATTTGAAACGTTATTTCTGCGAGTGTATCAAAATTTTATTTGGGGCAAGCGATACACCTTTACATTTTGTGAGGATCTCTATGTTGCCTTCAT 2514
 2515 CTAACCTCATTTTGTGGTCTACTCTGTAGGTGGCATGAACCTCAGTCTTACCCTGGCAATGCTGGTGGACGATTGGCATGCGTAAGAAGAAACACTCT 2614
 G G H E L S L T T G N A G G R L A C
 2615 TTTCTCCCTTTCTGCTGCGACTCTTCTCTCATGTATAATTACTAGTCTGACATTTTCTGCTTAAATGAAACAAACAATAACTACTCGGTGGAG 2714
 2715 AGTGTAGTCCCAAGAGTGAAGTTTGGGAGCGGTGTACGAGACCTTATCTCTACCGTACCTTATAGGTGAGAAATTTCTGAGACCTCGCTCAAGGAAA 2814
 2815 ACAGTCAAGCAGTCAGGAAAGAGTACAGTACACGACCTACAAGAAACAATTTCTGTCATCGAGGTACAAGAAATCAACAATAGGAACAGAAATCTAAGG 2914
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 3015 AAATTAGTAGAACTTAGCCTCGTATAGTTTAAACCCCTAGTTGCATTTGTGTTTATAATGGTTAAGATAATCTTAAGAGTTCGTTTGAAGTCTAGACTTT 3114
 3115 TGGCTGGGAATAATGTTATTTTAGCAAAAAGAAAGGACATAAGACAATAAAGTAAATGGTCCCTCATTTTCAAGTTGAATATGTGGTGCATTTTGTGTTG 3214

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3215 GTTAATGTCTTCTGTATACAGCTAGTTTGCCTTCTCTCCTTTTGTAGTTTAGCAGCAATTATACAATTATAACGTTTATATATATATTTATACACAAC 3314
3315 AACCAACAAACCTAGTGCATACCCACAGTGGGTACTACGGGAGGTAGGTATTTACACAGACATTATCCTTCTAG//GTTAAGGAGATCTATCGTCCCC 4767
4768 ATATGAGCGGTTAAACAAATTACGTCCCATATTTACTCATGCTCTGTGCATCTAGTGAAGGTTCAAATTCGTGTGCCATTATGCGAGGTTTAAACAAAG 4867
4868 CACGCATAAGAGAGACCTTTCAAGTTTGTGTAAAAGAGTGAGTTTGAAGCTAGCTCCAACTCCAACATCCATCACCATCCACCAGGTGCCAGCCCTGA 4967
4968 AAACATCCCCGCTCTTCTCGCTAAGTAGTTCATCTCCATGCCCCCTCTCCATTACCCGAGACAGATTATTTTGGGGTTTACTTGTATGTATGTAAGAA 5067
5068 AATACTTTTGAACGACATTGCTTTCGGTATATAGTTTGTCTGACTTTGATATGTCAAGAGGAAGACTAGATTACAAGTTCTTAGAAGTATCATATATAGC 5167
5168 TATGTGACTTACTCCACTTTGACTTGTAAATTCGTTCAAGTTGAGATCTTTCCTCTACTTTGGATTTCCTAAATGCTAGATATTTTCTCTTTATACTTT 5267
5268 CAGATGAGAGGCTTCTCGTGTACCTTCTTGGTCGGATTCTTACATGCTGTTTACAAACTACCTTTTCACTTATGTTTCATCGCGAAATTTTCCTATGC 5367
5368 AGGTGTGGTTGGTTTGAATCCCAATATGAAGTAATTCAGCAGCAACAACAGCTCTTTTGTGCGAATTATAATGAGTGAACGTATTTACCTCTCTCCGG 5467
    G V V G L T P I *
5468 GTTGCCAATAAACCTCTACATGAATTTGTACTGAGAAATCTTTGATGAACCTAAATTTGAACCTGTATGTACACACTTGCCAACTCTTTTGTCTCAA 5567
5568 ACTGTAATTTGGAGTTGGCTATTTACCTTCCTTTTGTGTTTGGCTATCTAAAGTTACACTCAGTTAACTAAGTGTGCTTGGATTTTGAACCTCTGTAT 5667
5668 GTATAGTTTCTCGAGACGAGGTCTATCAGAAACAACAATCTGTATGATTAAGTACAGATTATTTTATCACTTCTCTGAGTTAGACTTTTCCAAAAA 5767
5768 TACATTTTACTCATCTGTAAAGATAGGGTAAGGTCTAGGGGCGTCATCGGTCGGTTTTCATATATTTGGTTCCGGTTATCGGTTTTCGGTTTAA 5867
5868 AATATTCTAACCAATAACAACCAATAAGAAACTTTTATCGGTTATCGGTTTATATATATCCCGGTCGATTCGGTTAATCATTAAAGAAAGAGATC 5967
5968 AAATAATATATAATAGTACGATGTTATAATTACATGTACCCTATCGCCAAACATAATATGAAAC 6034

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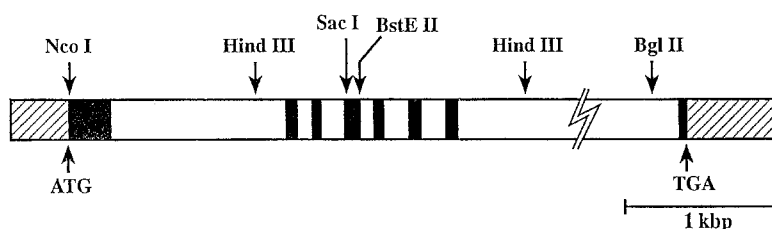


Fig. 2. A. Nucleotide sequence and derived amino acid sequence of the tomato *SODCp;Le:1* gene that codes for the chloroplastic Cu,Zn SOD. The transcriptional initiation site is marked by a vertical arrow and the putative TATA box is underlined. B. Scheme of *SODCp;Le:1*. The 442 bp, upstream of the translation initiation codon ('short promoter'-CSP) and the sequence downstream of the TGA stop codon are shaded; the exons are black and the introns are white. About 1 kb of the 7th intron was not sequenced.

activity assays. Samples were loaded on a non-denaturing 10% PAGE [16], run at 4 °C (6 V/cm⁻¹) and stained by the riboflavin-nitrobluetetrazolium method [4].

Southern and northern blot analysis

Plant DNA extraction and Southern blot analysis were performed as described by Galili *et al.* [8], with the exception of using Hybond-N⁺ membranes (Amersham). A 2 kb *Eco* RI-*Bam* HI fragment from pBI101 that contains the *uidA* gene (GUS), was labelled with ³²P and served as a probe.

Total RNA extraction from plant samples and electrophoresis was performed according to

Perl-Treves and Galun [21]. RNA was blotted onto Hybond-N⁺ or Hybond-N membranes (Amersham) and hybridized with a ³²P-labelled 700 bp PCR fragment from the PBI101 plasmid that contained part of the *uidA* gene. The blot was washed twice at room temperature for 15 min with 1 × SSC, 0.2% SDS, before exposure on X-ray film.

Results

Characterization of the chloroplastic Cu,Zn SOD gene

In order to characterize the chloroplastic Cu,Zn SOD gene and to study the regulatory capacity of

its promoter, we screened a genomic library of tomato with the respective cDNA as probe. The resulting DNA fragment is shown in Fig. 2 and termed *SODCp;Le:1*. Figure 2A details the nucleotide sequence and the derived amino-acid sequence coded by the exons and Fig. 2B provides the schematic pattern of the chloroplastic Cu,Zn SOD gene of tomato. The gene is composed of 8 exons interspaced by 2 long (the 1st and the 7th) and 5 short introns. This tomato gene has a general similarity to the gene coding for cytosolic Cu,Zn SOD of rice [24]; the latter is also composed of 8 exons and 7 introns. Since there is no previous report on a chloroplastic Cu,Zn SOD gene, the tomato gene cannot be compared to respective genes in other plants. The nucleotide sequences in the exons in this tomato gene are fully homologous with the previously sequenced cDNA that codes for the chloroplastic Cu,Zn SOD of tomato [22]. The presently isolated genomic clone of tomato has 442 bp upstream of the translational initiation site. The latter fragment contains a putative TATA box (underlined in Fig. 2A). The transcriptional initiation site (marked by a vertical arrow) was determined by primer extension analysis (not shown); it was found to be 157 bp upstream of the translational initiation site. The remaining upstream promoter fragment spans 285 bp upstream of the transcriptional initiation. For brevity we termed the 442 bp fragment CSP (for chloroplastic Cu,Zn SOD short promoter).

Transgenic plants with the GUS gene driven by the CSP

The 442 bp CSP fragment was cloned into the pBI101 vector, upstream of the *uidA* (GUS) reporter gene (causing a transcriptional fusion between CSP and GUS). The engineered vector (Fig. 1) was introduced into *Agrobacterium tumefaciens* cells (LBA 4404) and the latter served to transform tobacco leaves. Shoots that regenerated from inoculated leaves and rooted on kanamycin-containing medium were considered putative transformants. The presence of the CSP::GUS fused gene, in some of the transformants,

was verified by Southern blot hybridization (Fig. 3).

Out of over 30 putative transformants, 21 reached the stage of greenhouse-grown transgenic plants and their young leaves were analyzed for GUS activity. Plants in which the GUS activity was above a threshold of 1 nmol/min per mg protein were grown to maturity and their seeds (resulting from self-pollination) were harvested. The respective seedlings that passed kanamycin selection were either transferred to the greenhouse for experiments with adult plants or transferred to Petri plates with solidified Nitsch medium for experiments with *in vitro* grown seedlings.

Different light/dark responses in transgenic seedlings

We previously found [21] that the transcript levels of the chloroplastic Cu,Zn SOD in young tomato

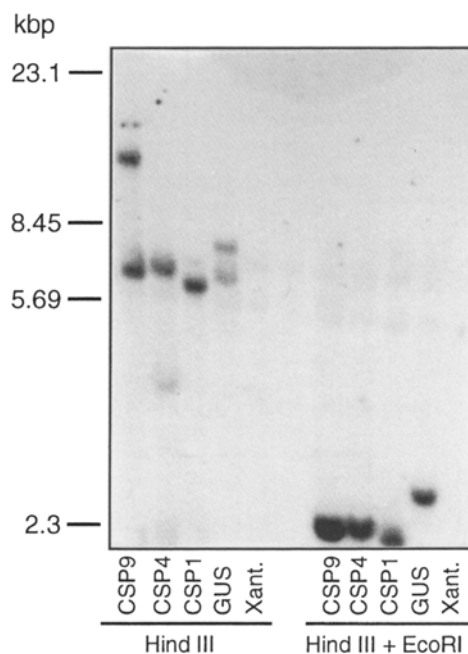


Fig. 3. Southern blot analysis of DNA from three transgenic lines. DNA samples (10 µg per lane) were digested with endonucleases, run on an agarous gel and the blot was hybridized with a ³²P-labelled GUS probe. GUS and Xant. denote positive and negative controls, respectively. The GUS control is DNA from a transgenic plant transformed with a different GUS-containing vector, therefore it has a slightly larger GUS insert than in the three tested lines.

plants is enhanced by light. To test whether or not the short promoter of the chloroplastic Cu,Zn SOD gene (CSP) will activate the reporter gene (GUS) we grew the transgenic seedlings for three weeks in a 16 h/8 h light/dark regime ($70\text{--}100\ \mu\text{E m}^{-2}\text{ s}^{-1}$; $25\ ^\circ\text{C}$). They were then transferred to 3 days of dark that were followed by either 8 h dark or 8 h light. Figure 4 shows that this light/dark screening resulted in two groups of transgenic plants. In the first group (A) the seedlings had either very low GUS activity after three days of dark (4 lines), or their GUS activity was relatively low. In all the 10 lines of this group 8 h of subsequent illumination did enhance GUS activity. This indicated that in the transgenic seedlings of group A the CSP promoter was indeed light-responsive. On the other hand, seedlings of group B had GUS activity when grown in light as well as in dark. Moreover one of the transgenic lines (CSP-7) had extremely high GUS activity after over 3 days of culture in the dark. We choose one line of group A (CSP-1) for further experiments.

Induction of GUS in CSP-1 by light and wounding

To study the light response in more detail we exposed CSP-1 seedlings to a light/dark regime

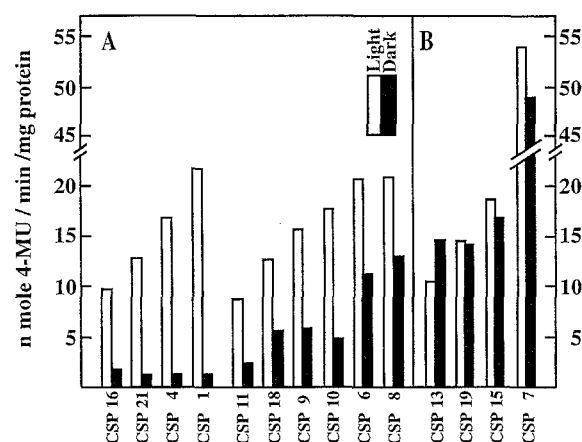


Fig. 4. Screening of transgenic seedlings for light-induced GUS activity. Seedlings were cultured for 3 days in the dark and then either cultured in the dark for additional 8 h or exposed to light for 8 h. The seedlings were then assayed for GUS activity. Note that the 14 CSP lines can be assembled into 2 groups.

schematized in Fig. 5A and followed the response of these seedlings by analyzing GUS activity as well as the endogenous SOD activity and the GUS transcript level (Fig. 5B, C and D). Again, GUS activity was negligible in seedlings grown for 3 days in the dark. They also had no

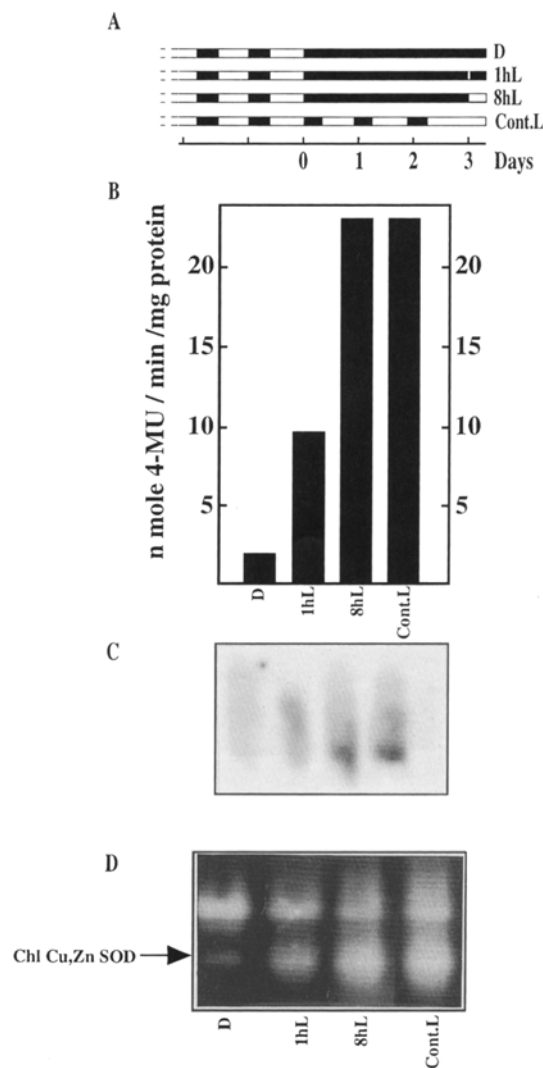


Fig. 5. The response of CSP-1 seedlings to different light regimes. A. Schemes of light/dark regimes; light and dark periods are represented by white and black sections, respectively. B. GUS activity in seedlings exposed to different light/dark regimes. C. Northern blot hybridization of the RNA from seedlings of B; $10\ \mu\text{g}$ total RNA were applied in each lane of an agarose gel and the blot was hybridized with a ^{32}P -labelled GUS probe. D. SOD activity gel with protein extracts from the seedlings of B; $80\ \mu\text{g}$ protein were loaded in each lane.

detectable endogenous chloroplastic Cu,Zn SOD activity and had very low GUS transcript. One hour of light (followed by 7 h dark) was sufficient to induce GUS activity as well as to show endogenous chloroplastic Cu,Zn SOD activity and GUS transcript. Exposure of the dark-grown seedlings to 8 h of light further increased the GUS activity to the same level of GUS activity as in 'continuous light'-grown seedlings.

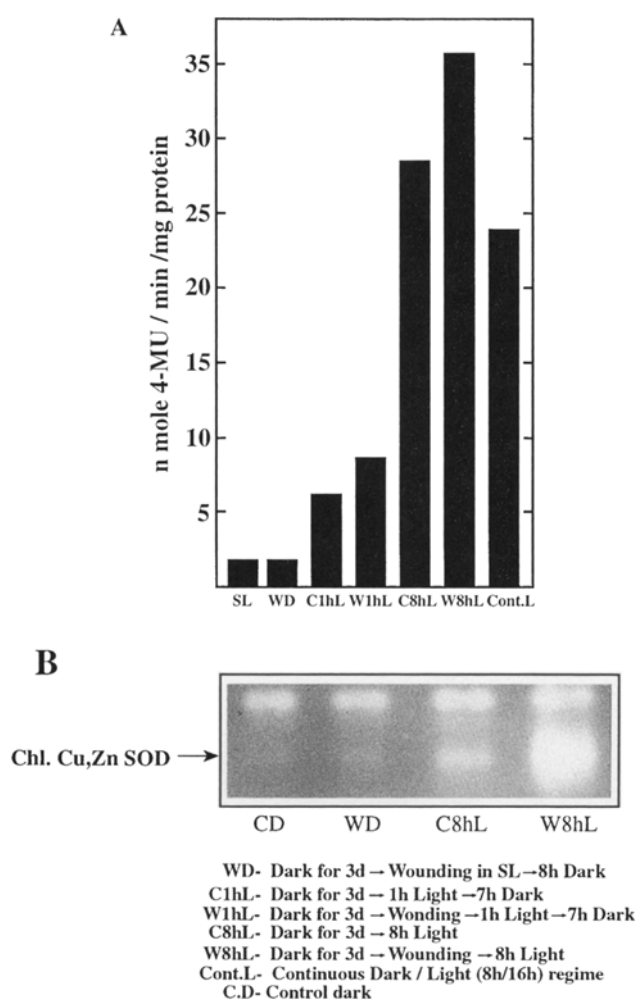


Fig. 6. The response of CSP-1 seedlings to light induction was wounding. See Fig. 5 for details of light/dark regimes. **A**, GUS activity. **B**, SOD activity gel, 80 μ g protein were loaded in each lane. SL is exposure for 5 min to pale-green safe-light in a dark-room (5 min are required to execute the wounding). CD, control, dark-grown seedlings. Note that the activity of the cytosolic Cu,Zn SOD (the upper band) is similar in all the four treatments.

We previously found [21] that wounding of tomato leaves increased the level of the mRNA for their Cu,Zn SOD. We therefore asked whether or not the short promoter of the chloroplastic Cu,Zn SOD is responsive to wounding. Consequently we combined wounding, by pricking the leaves of CSP-1 seedlings, with different light/dark regimes that followed 3 days of culture in the dark (Fig. 6). GUS activity (Fig. 6A) was apparently further enhanced by wounding in seedlings exposed for 1 h to light (followed by 7 h dark) as well as in seedlings exposed to 8 h light. Very low GUS activity was detected in seedlings that were wounded in safe-light and returned to dark. The activity of endogenous chloroplastic Cu,Zn SOD (Fig. 6B) is higher in seedlings exposed to light (C8hL) than in dark-grown seedlings (CD); wounding (W8hL) increased this SOD activity several-fold over illuminated seedlings that were not wounded (C8hL).

Developmental regulation

In tomato plants the expression of the gene for chloroplastic Cu,Zn SOD is clearly developmentally regulated [21, 22]. In order to find out whether or not the short promoter of this gene has a spatially regulatory capacity we grew CSP-1 plants in the greenhouse and analyzed leaves of three developmental stages as well as the roots for GUS activity, GUS transcript level and the endogenous SOD level (Fig. 7). Notably, very low GUS levels were detected in mature leaves and very low GUS activity was found in the roots. These levels were in direct correlation with the endogenous chloroplastic Cu,Zn SOD levels in these plant tissues. It is also noteworthy that while no GUS activity was detected in the roots of CSP-1 plants the roots of CSP-7 seedlings contained high levels of GUS (not shown). The results (Fig. 7) indicated that while the GUS transcript level was higher in young leaves than in expanding leaves, the GUS activity and the endogenous chloroplastic Cu,Zn SOD were not higher in young leaves than in expanding leaves.

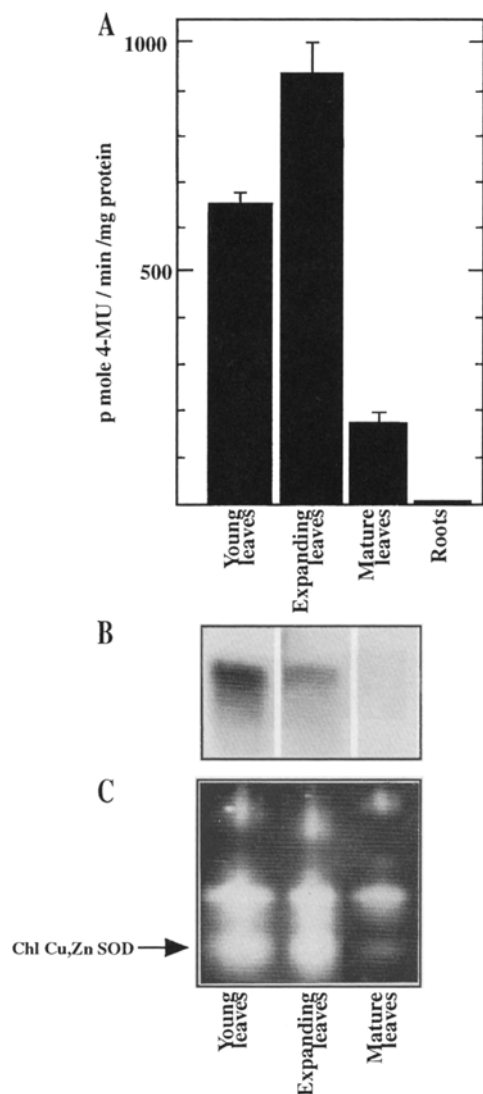


Fig. 7. Developmental and spatial expression of GUS in CSP-1. **A.** GUS expression in roots and in leaves of three developmental stages; the histograms are means of two plants, the range is provided by the vertical bars. **B.** Northern blot hybridization of RNA from leaves of three developmental stages; 10 μ g total RNA were run in each lane of an agarose gel and the blot was hybridized with a ^{32} P-labelled GUS probe. **C.** SOD activity gel with protein extracts from leaves; 80 μ g protein was loaded in each lane.

Discussion

The *SODCp;Le:1* gene

We isolated and sequenced a genomic clone (*SODCp;Le:1*) that includes all the exons and in-

trons of the gene encoding chloroplastic Cu,Zn SOD as well as part of its promoter. The latter's *cis*-regulatory elements are of great interest because the gene is activated by internal signals (e.g. in specific plant organs, at specific stages of fruit and leaf development) as well as by a plethora of external effectors [see 8]. The nucleotide sequences of the exons of the genomic DNA clone isolated by us (Fig. 2) are homologous to those of the cDNA for the chloroplastic Cu,Zn SOD of tomato [22]. Previous data indicated that there is probably only one gene in tomato that codes for this enzyme [20, 22], so there is probably only one promoter that regulates the expression of *SODCp;Le:1*. The flanking nucleotides of the translation-initiation codon in *SODCp;Le:1* (GC-CATGGC) resemble those of vertebrate genes and some plant genes (e.g. A/GCCATGGC [7] and ACCATGG [13]). The *SODCp;Le:1* gene has a 157 bp leader upstream of the translational start and a conserved TATA box that starts 34 bp upstream of the transcriptional initiation site. We did not find *cis* motifs in the CSP of *SODCp;Le:1* having homology to reported *cis* motifs in plants that are involved in gene regulation by light [see 17].

Light-regulated and 'constitutive' GUS activity

The results of this study showed that in most, but not in all, transgenic plant lines, GUS expression was suppressed by culture in the dark. In 4 out of 14 tested plant lines GUS activity was reduced in the dark to background levels but this activity was induced after a short light exposure. In 4 other plant lines (Fig. 4, group B) dark/light regimes had no effect on GUS expression. One of these lines had a relatively high expression of GUS and even its roots contained GUS activity. These results are compatible with those of Topping *et al.* [30] and Lindsey *et al.* [18]. These authors applied transformation with the GUS gene (*uidA*), either fused to a very short promoter or without a promoter, to tag genomic sequences in transgenic plants. They found that the expression of GUS was indeed site-specific and that

such transformations were useful to investigate position effects and for tagging genes in developmental studies. It is thus plausible that the fused gene used by us, having a relatively short promoter, can also be strongly affected by the position of its insertion: when inserted downstream of a strong enhancer motif, GUS will be 'constitutively' expressed regardless of its CSP.

Regulatory capacity of CSP

Experiments with seedlings of one of the transgenic lines (CSP-1) in which GUS expression was modulated by dark/light regimes, indicated that even one hour of light (followed by 7 h of dark) was sufficient to induce GUS expression. Indeed 8 h of light brought GUS expression to the maximum level of 'continuous light'. Furthermore, the *uidA* transcript increased in parallel to the increase of GUS activity. Also, the endogenous activities of chloroplastic Cu,Zn SOD was enhanced by the same dark/light regimes. Transgenic plants of the line CSP-1 showed also developmental and spatial regulation of GUS activity: high activity in young leaves, very little activity in mature leaves and no activity in their roots. It is noteworthy that while CSP-1 seedlings were highly induced by light, they had apparently only a single insertion of the transgene (Fig. 3). Our results with wounding indicate that there was no GUS enhancement in dark-grown transgenic seedlings (of CSP-1), but wounding in combination with light probably increased (by about 25%) GUS expression over the same light treatments that were provided to seedlings that were not wounded. The endogenous levels of chloroplastic Cu,Zn SOD were increased several-fold by wounding of illuminated transgenic seedlings. This much greater increase in activity of the endogenous SOD than the GUS activity suggests that the CSP either does not contain the wounding-responding motifs of the whole chloroplastic Cu,Zn SOD promoter or CSP is only partially responding to wounding.

We attributed changes in GUS activity to the dark/light regimes. But it is possible that the light

has an indirect effect: the CSP could be sensitive to O_2^- that results from light-driven photosystem II. Further physiological studies may clarify this point.

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