A Comparison of the Structure and Function of the Highly Homologous Maize Antioxidant Cu/Zn Superoxide Dismutase Genes, *Sod4* and *Sod4A*

Sheri P. Kernodle and John G. Scandalios

Department of Genetics, North Carolina State University, Raleigh, North Carolina 27695-7614

Manuscript received April 22, 1996
Accepted for publication June 7, 1996

ABSTRACT

Two highly similar cytosolic Cu/Zn *Sod* (*Sod4* and *Sod4A*) genes have been isolated from maize. *Sod4A* contains eight exons and seven introns. The *Sod4* partial sequence contains five introns. The introns in both genes are located in the same position and have highly homologous sequences in several regions. The largest intron (>1200 bp) interrupts the 5′ leader sequence. The presence of different regulatory motifs in the promoter region of each gene may indicate distinct responses to various conditions. Zymogram and RNA blot analyses show that *Sod4* and *Sod4A* are expressed in all tissues of the maize plant. The developmental profiles of *Sod4* and *Sod4A* mRNA accumulation differ in scutella during sporophytic development. RNA blot analysis of the respective *Sod* mRNAs indicates a differential, tissue-specific response of each gene to certain stressors. RNA isolated from stem tissue of ethephon-treated seedlings shows an increase in the *Sod4* but not the *Sod4A* transcript while there is no change in transcripts of either gene in leaves or roots. There is differential mRNA accumulation between the two genes in leaf and stem tissue of paraquat-treated seedlings. Other agents that can cause oxidative stress were also tested for differential expression of the genes.

SUPEROXIDE dismutases (superoxide:superoxide oxidoreductase; EC 1.15.1.1; SOD) are ubiquitous enzymes among all aerobes and are involved in protecting against oxygen toxicity (FRIDOVICH 1978). These enzymes catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide. Superoxide radicals ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) can damage molecular (nucleic acids, proteins, lipids) as well as cellular (membranes and organelles) components (SCANDALIOS 1993), and have been implicated in the cause of some human diseases.

SODs have been classified into three types based on the metal(s) contained: Mn, Fe, and Cu/Zn. The Mn and Fe SODs are both found in prokaryotes while the Mn SOD is found in the mitochondria of eukaryotes. The Mn and Fe SODs are structurally related to each other but differ from the Cu/Zn SODs. The Cu/Zn SODs are found in the chloroplast and cytosol of eukaryotes and in some prokaryotes (FRIDOVICH 1995). The presence of Fe-containing SODs has been reported in some plant species (KANEMATSU and ASADA 1990).

In maize the nuclear-encoded metalloprotein superoxide dismutase consists of nine isozymes: (a) four Cu/Zn cytosolic isozymes (SOD-2, SOD-4, SOD-4A, and SOD-5), (b) a small family of four mitochondrial associated MnSOD isozymes (SOD-3.1, -3.2, -3.3, -3.4), and (c) a Cu/Zn chloroplast associated isozyme (SOD-1) (BAUM and SCANDALIOS 1979; CANNON *et al.* 1987; CANNON and SCANDALIOS 1989; ZHU and SCANDALIOS 1993).

Corresponding author: John G. Scandalios, Department of Genetics, Box 7614, North Carolina State University, Raleigh, NC 27695-7614.

The cytosolic isozyme SOD-4A is similar to SOD-4 and is biochemically indistinct from SOD-4. Originally, the SOD-4 protein was defined as a single band of activity by zymogram analysis (BAUM and SCANDALIOS 1979). The N-terminal sequence for the first 20 amino acid residues was determined for the SOD-4 protein. The only discrepancy in the amino acid sequence occurred at amino acid 12 where both glutamic and aspartic acids occur. The deduced amino acid sequence obtained after DNA sequence analysis of the cDNA clones showed that SOD-4 contains glutamic acid while SOD-4A contains aspartic acid at that position. The remainder of the deduced amino acid sequences differ at only one other residue (amino acid 29). The DNA sequences of *Sod4* and *Sod4A* are 95% homologous in the coding region. Genomic DNA blots probed with gene-specific sequences (GSS) confirmed that two genes exist (CANNON and SCANDALIOS 1989).

The present report focuses on the isolation and characterization of the *Sod4* and *Sod4A* genes and a comparison to each other and to other superoxide dismutase genes which have been isolated. RNA blot analysis has indicated that the two genes respond differently to certain stressors but not to others. The response of these two highly conserved maize genes to the various agents known to cause oxidative stress as well as their expression during development was examined.

MATERIALS AND METHODS

Seed germination: Seeds of the maize inbred line W64A (*Zea mays* L.) were surface sterilized for 10 min in 1% sodium

hypochlorite, rinsed, and soaked in deionized water for 24 hr. Seeds were then either germinated in continuous darkness on moistened Kimpak germination paper at 25° or planted in flats containing Metromix potting soil and grown using a 12/12 hr light/dark cycle.

Library preparation, screening and subcloning: *Sod4.* A genomic DNA library was constructed from 14 days post-imbibition (dpi) greenhouse grown leaves by cell lysis in a buffer containing 2% *N*-lauryl sarcosine, 0.2% β-mercaptoethanol, 30 mM EDTA pH 8.0, 50 mM Tris pH 8.0, and 300 μg/ml ethidium bromide followed by cesium chloride centrifugation and dialysis in 1× TE pH 8.0 (10 mM Tris, 1 mM EDTA). The DNA was partially digested with varying concentrations of *Sau*3A to determine which concentration gave the maximum number of DNA fragments between 15 and 20 kb. Size selected DNA was obtained by sucrose gradient centrifugation. The vector λGEM-11 (Promega Biotech) was prepared according to manufacturer's directions. The genomic DNA and vector were ligated together, packaged (Stratagene) and titered on LE 392 cells. Duplicate nitrocellulose filters were prepared then denatured (0.5 M NaOH, 1.5 M NaCl), neutralized (1.0 M Tris pH 8.0, 1.5 M NaCl) and rinsed in 2× SSC. Filters were baked for two hours at 70° in a vacuum oven to bind the DNA to the filter. After prewashing (50 mM Tris pH 8.0, 1 M NaCl, 1 mM EDTA pH 8.0, 0.1% sodium dodecyl sulfate) to remove excess cellular debris, filters were prehybridized and hybridized in a solution containing 6× SSC, 5× Denhardt's, 0.05 M sodium phosphate pH 7.0, 0.1% sodium dodecyl sulfate (SDS), 200 μg/ml sheared and denatured salmon sperm DNA, and 50% formamide at 42° for 16–18 hr. *Sod4* and *Sod4A* full length cDNAs were radioactively labeled with [α-^{32}P]dCTP (FEINBERG and VOGELSTEIN 1983). Filters were sequentially washed in 5× SSC, 0.1% SDS at 23°; 2× SSC, 0.1% SDS at 42°; and 0.1× SSC, 0.1% SDS at 60°. Filters were air dried and exposed to Kodak XAR film. Positive plaques were isolated and purified to homogeneity (MANIATIS *et al.* 1982).

Sod4A. A size-selected genomic DNA library from line W64A was prepared. Total DNA isolation was as described above. The DNA was restricted with *Eco*RI and electrophoresed on a 0.7% agarose gel. The region that contained the size desired was excised and the DNA electroeluted using an Isco Concentrator model 1750 according to manufacturer's directions. The DNA was ligated to the *Eco*RI site of the lambda replacement vector EMBL4 (Stratagene), packaged, titered on LE 392 cells and duplicate nitrocellulose filters were prepared and probed as described above.

Phage and plasmid DNA isolation: Phage DNA from the positive plaques was isolated by a modified M13 phage DNA isolation procedure (KERNODLE *et al.* 1993). Plasmid DNA was isolated by an alkaline lysis method (BIRNBOIM and DOLY 1979).

Southern blot analysis: Plasmid DNA was digested with restriction enzymes (Boehringer Mannheim) and electrophoresed on agarose gels of varying concentrations depending on the size of the DNA. Denaturing, neutralizing, and transfer to nitrocellulose was as described (MANIATIS *et al.* 1982). Hybridizations and autoradiography were performed as described earlier.

Sequencing: Single-stranded M13 DNA or double-stranded plasmid DNA were used as templates. Small DNA fragments (<500 bp) were sequenced from both directions. Larger fragments (>1 kb) were subcloned into pBluescript and nested deletions made using Nuclease S1 (Stratagene). The Sanger dideoxy method was used for all sequencing (SANGER *et al.* 1977).

Primer extension: Primers for *Sod4* (5′TCCAGAAGCGAAGACTC3′) and *Sod4A* (5′TAAGGCCGTCGCCTTCCCATT3′) were kinased at 37° for 30 min followed by heating to 70° for 10 min to denature the enzyme. The reactions were purified on Sephadex G50-50 (20–50 μm) columns and counted on a Beckman LS6000 scintillation counter. The primers were diluted to approximately 0.002 pmol/μl in 1× reverse transcriptase buffer (Boehringer Mannheim). Ten, 20 and 50 μg of total W64A RNA from scutella were lyophilized. Approximately 60 fmol of primer was added to each RNA sample, heated to 65°, vortexed briefly to resuspend and returned to 65°. The heating block was turned down to 37° and the annealing proceeded overnight. The extension reaction was incubated at 37° for 30 min followed by phenol/chloroform extraction and ethanol precipitation. The pellets were resuspended in loading buffer of deionized formamide containing 0.3% xylene cyanol FF, 0.3% bromophenol blue and 0.37% EDTA pH 7.0. The extension products and a sequencing ladder, using appropriate primers, were run on a 6% acrylamide/8M urea gel. The gel was then dried and exposed to XAR-film.

Temperature and chemical agent treatments: The maize inbred line W64A was used for all treatments. Seeds were germinated and grown at 25, 35 and 40° and scutella collected at 1–10 dpi.

Greenhouse grown seedlings (12 dpi) were treated with increasing concentrations of ethephon or paraquat for 17 hours by hydroponic uptake. Leaf, stem, and root tissues were harvested, frozen in liquid nitrogen and stored at −80° until used.

Embryos isolated from kernels at 21 and 28 days postpollination (dpp) were placed on Murashige and Skoog (MS) media plates with basal salts plus B-5 vitamin containing various concentrations of hydrogen peroxide (H_2O_2) ranging from 0 to 0.1%. Plates were placed in the light or dark at 25° for 24 hr. Two and 3 dpi or 25 and 28 dpp embryos were treated with 0–5 mM salicylic acid in the same manner as for H_2O_2. The tissue was harvested, frozen, and stored as described.

RNA isolation and blot analysis: Total RNA was extracted from maize tissues by a cold phenol extraction method (BEACHY *et al.* 1985). Total RNA was electrophoresed through 1.2% agarose gels containing formaldehyde and transferred to nitrocellulose (MANIATIS *et al.* 1982). DNA fragments were labeled as described. Prehybridization and hybridization were performed as earlier described. The *Sod4* GSS or *Sod4A* GSS (CANNON and SCANDALIOS 1989) were used as probes at a concentration of 2×10^6 cpm/ml at 42° for 24–48 hr. Filters were washed two times with 2× SSC, 0.1% SDS at 42°, followed by one wash with 0.1× SSC, 0.1% SDS at 65°, air dried and exposed to Kodak XAR film. The 8.8-kb fragment of pea ribosomal DNA (JORGENSEN *et al.* 1987) was used as a loading control on all RNA hybridizations.

RESULTS

Cloning, structure and sequence analysis of the *Sod4* and *Sod4A* genes: The *Sod4* gene was isolated from *Sau*3A digested genomic DNA which was size selected (~15–20 kb insert length) on a sucrose gradient and ligated to the vector λGEM-11. The full-length *Sod4* cDNA was used to probe the library. Two putative positives were isolated, one of which was *Sod4*. Subsequent restriction digestion and Southern analysis placed the *Sod4* gene on a 9-kb *Sal*I fragment. The *Sod4A* gene was isolated from a library constructed by digesting genomic DNA with *Eco*RI, electrophoresing the DNA through a 0.7% agarose gel and excising a region that

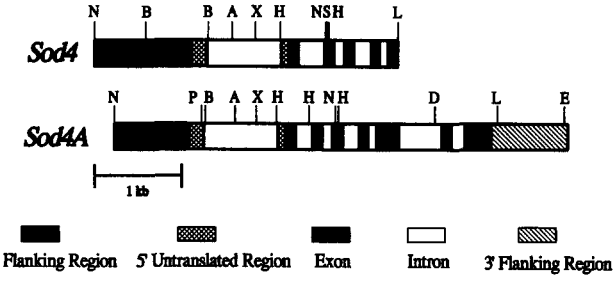

FIGURE 1.—Structural maps of the *Sod4* and *Sod4A* genes. Orientation was determined after hybridization with the 5′, middle, and 3′ sequences of the *Sod4* cDNA. The *Sod4A* gene has eight exons and seven introns. The partial *Sod4* gene has six exons and five introns. The two genes have similar exon and intron placement and size. The 5′ leader sequence is interrupted by a large intron (>1200 bp) in both genes. A, *Acc*I; B, *Bam*HI; D, *Hin*dIII; E, *Eco*RI; H, *Hin*dII, L, *Sal*I, N, *Nco*I, P, *Pst*I; S, *Sph*I; X, *Xba*I.

contained the desired DNA size. The electroeluted DNA was then ligated to the lambda replacement vector EMBL4. This library was probed with the *Sod4A* cDNA. Two putative positives were obtained. The *Sod4A* gene was located on a 15-kb *Eco*RI fragment after Southern analysis. Two libraries were screened to obtain the genes because both genes were not isolated from the initial library.

The *Sod4* 9 kb *Sal*I and *Sod4A* 15-kb *Eco*RI fragments were subcloned into pUC12, digested with various restriction enzymes, and the structural maps determined for *Sod4* and *Sod4A* (Figure 1). Southern blot analysis with the full length cDNAs was used to determine which restriction fragments of the genomic clones contained sequences complementary to the cDNAs. The *Sod4* cDNA hybridized to an 806-bp *Bam*HI fragment and a 2.4-kb *Bam*HI/*Sal*I fragment. The *Sod4A* cDNA hybridized to a 118-bp *Pst*I/*Bam*HI and a 3-kb *Bam*HI/*Hin*dIII fragment. The fact that each cDNA mapped to two genomic fragments, one of which was very large, indicated that each genomic clone probably contained at least one intron. This was confirmed when the 5′, coding, and 3′ regions of the *Sod4* cDNA were used as probes. The 5′ region indicated not only orientation of the genes but also confirmed that the 5′ leader sequence of each gene was located on two restriction fragments. Hybridization using the coding region showed that the *Sod4* coding region was located on three restriction fragments while that of *Sod4A* was located on four restriction fragments. The 3′ untranslated region of each gene is positioned on a single restriction fragment.

The nucleotide sequences were characterized for both *Sod4* (GenBank accession no. U34726) and *Sod4A* (U34727) (Figure 2). Analysis of the sequence data indicates numerous similarities between the two genes. There are eight exons and seven introns in the *Sod4A* gene. The *Sod4* clone contains the promoter and the first 125 amino acids. Due to the way the library was constructed (*Sau*3A digestion) and the presence of several *Sau*3A sites in the 3′ region of the *Sod4* cDNA, only a partial clone was obtained. The location of the introns is identical in both genes and the size of each corresponding intron is fairly similar with the exception of the second intron (Table I). Both genes contain a very large intron interrupting the 5′ leader sequence. All introns have the GT . . . AG consensus sequence for splicing junctions (BREATHNACH and CHAMBON 1981). In addition the 5′ and 3′ splice junction sequences are similar between *Sod4* and *Sod4A*. For example, both the 5′ and 3′ splice junction sequences of the second intron are identical except for the last base of the 5′ junction, which for *Sod4* is an A and for *Sod4A* is a C.

There are many similarities between *Sod4* and *Sod4A* with respect to their introns. Some of the corresponding introns contain some identical sequences. In the first intron which interrupts the 5′ leader sequence, there are several regions that are similar, particularly around the *Acc*I and *Xba*I sites, indicating that these sites may be conserved. There are also some areas of sequence identity in the second intron. The third and fourth intron sequences are almost completely identical. Intron three is 74% and intron four is 89% identical between the two genes.

Promoter analysis: Promoter and 5′ leader sequences prior to the intervening sequence were analyzed to determine the location of the transcription start site (ts), CAAT and TATA boxes and the presence of regulatory motifs (Figure 3). Location of the transcription start of *Sod4* and *Sod4A* by primer extension is indicated by +1. Motifs placed on the line are located on the sense strand and occasionally on both sense and antisense strands (*e.g.*, ACGT, bHLH) while those placed below the line are located on the antisense strand. Relative position of the motifs is indicated by the numbers below the line. All motifs present are shown to underscore differences in the two promoters, irrespective of potential functional significance. For example, the ABA responsive element (MARCOTTE *et al.* 1989) located in the *Sod4* promoter is 335 bp upstream of the ts. Generally this element is located approximately 150 bp upstream of the ts in most ABA response genes. Although this sequence is distally located from the ts in *Sod4,* preliminary RNA blot results have indicated that ABA does have an effect on the expression of *Sod4* but not *Sod4A* (L. GUAN and J. G. SCANDALIOS, unpublished results). The motifs, their sequence, response, and location in *Sod4* or *Sod4A* are listed in Table 2. The two genes share some of the same elements [ACGT (TABATA *et al.* 1989; ARMSTRONG *et al.* 1992), AP-1 (MEYER *et al.* 1993), bHLH (PABO and SAUER 1992), GT-1 (GREEN *et al.* 1988; TERZAGHI and CASHMORE 1995), and I-box (TERZAGHI and CASHMORE 1995)]. *Sod4* also contains the following elements: ABA, G-box (light) (GIULIANO *et al.* 1988), Gap-box (light) (CONLEY *et al.* 1994), SV-40 (enhancer) (HATZOPOULOS *et al.* 1988) and wound (wounding) (PABO and SAUER 1992)

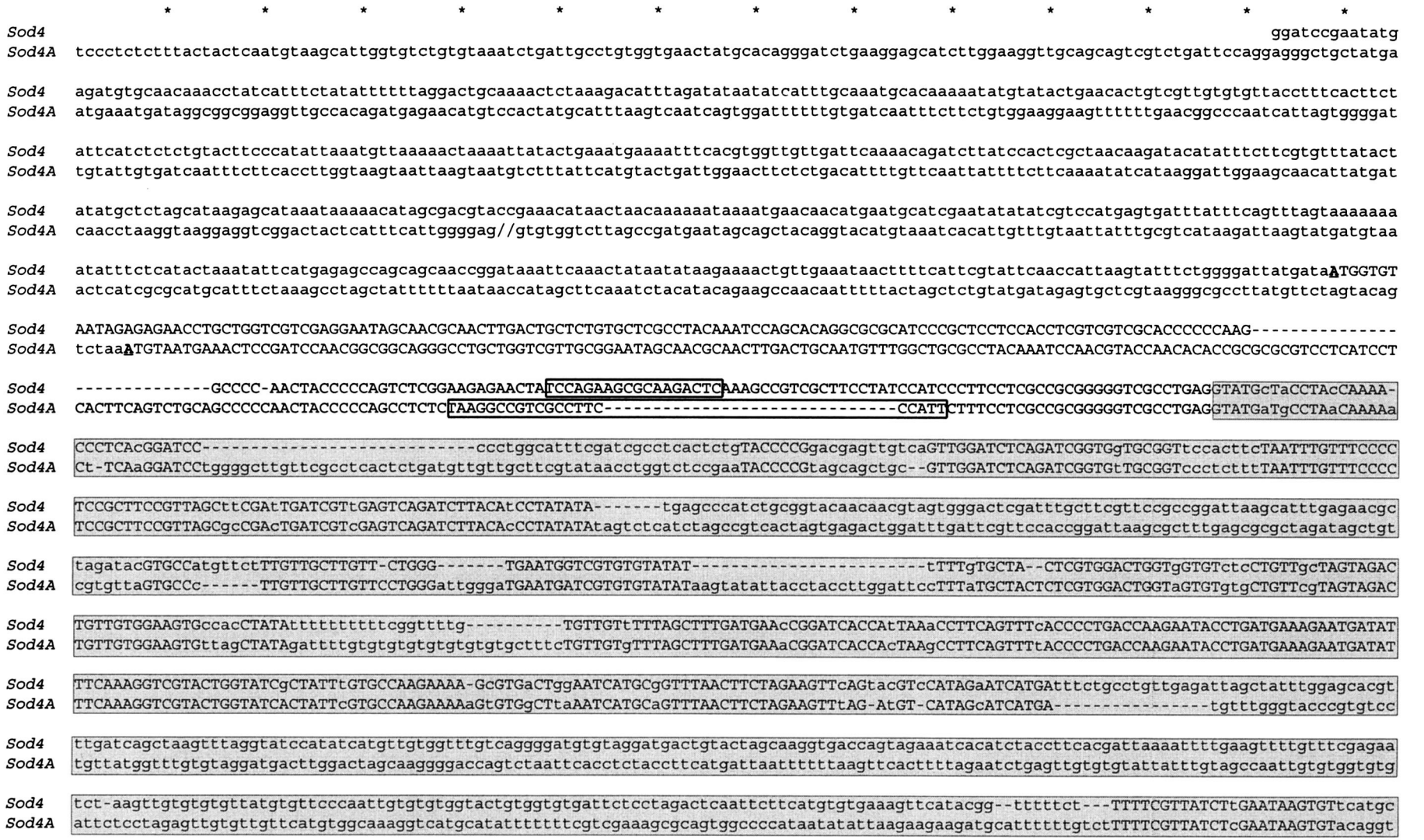

FIGURE 2.—Nucleotide sequence of the *Sod4* and *Sod4A* genes. The deduced amino acid sequence is indicated above the nucleotide sequence. Upper case letters denote the transcribed sequences of the genes, while lower case letters denote sequence before the start of transcription and after the end of the genes. The bold, underlined A's indicate the start of transcription for the two genes. The primers used in primer extension are boxed in. Intron sequences are denoted by the shaded areas and capital letters within these areas indicate sequences identical between the two genes. The polyadenylation signal for *Sod4A* is underlined. The asterisk at the end of the *Sod4* sequence indicates the end of the truncated clone. 'End' is placed at the end of the *Sod4A* amino acid sequence. Dashes (-) in the sequence are placed where there is no corresponding base(s) in the sequence. The slashes (//) indicate a break in the sequence. The asterisks (*) at the top of the sequence are placed every 10 bp.

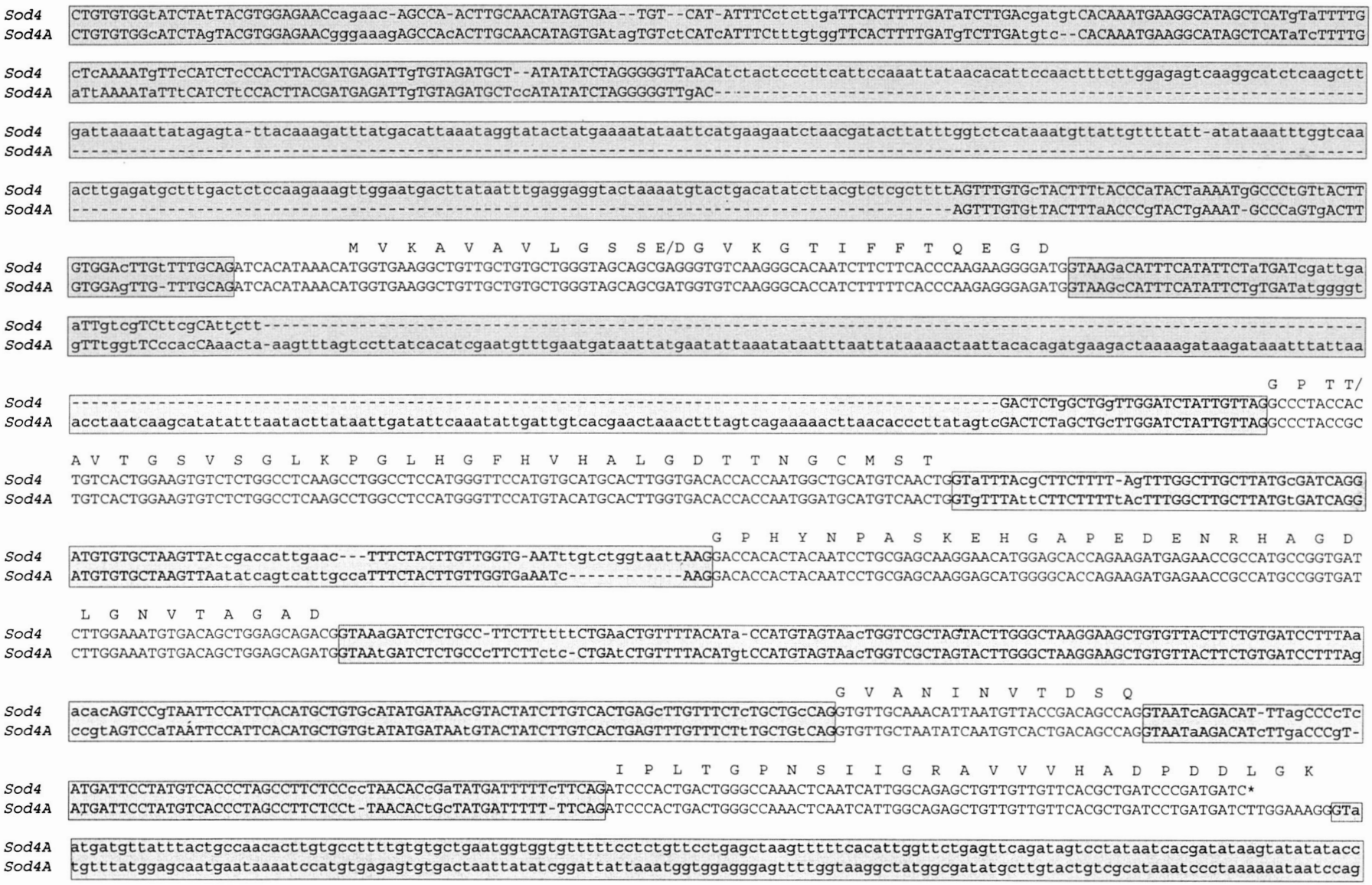

FIGURE 2.— *Continued*

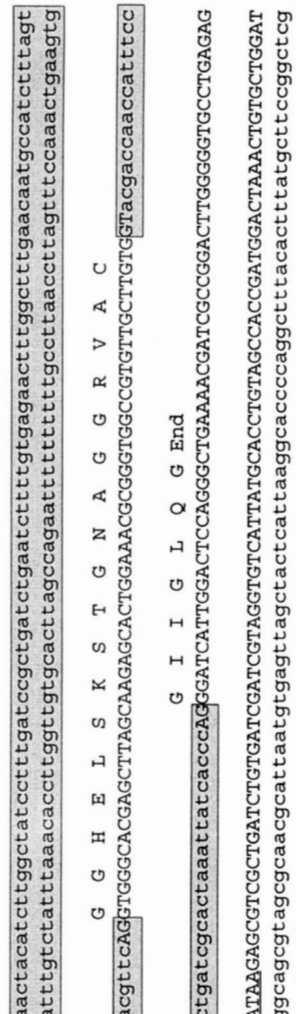

FIGURE 2.—*Continued*

while *Sod4A* contains bzip-hexamer (leucine zipper factors) (KATAGIRI *et al.* 1989), cAMP-receptor (receptor) (MONTMINY *et al.* 1986), two different heat shock elements (PELHAM 1982; SORGER 1991), Myb binding site (regulation of phenylpropanoid products) (SABLOWSKI *et al.* 1994), an 8-bp octamer (enhancer) (HARVEY *et al.* 1982) and three Y-boxes (cold stress response, enhancer) (WOLFFE 1994). The antioxidant response element (RGTGACNNNGC) is not present in either gene (RUSHMORE *et al.* 1991). An ethylene binding response element (TAAGAGCCGCC) was also not found in either *Sod4* or *Sod4A* (OHME-TAKAGI and SHINSHI 1995). RNA blot analysis of leaves and stems from seedlings treated with ethephon showed that *Sod4* transcript levels increased in response to elevated levels of ethephon while those of *Sod4A* do not. Computer analysis of the *Sod4* and *Sod4A* DNA sequences did not reveal the presence of any other reported regulatory motifs. The presence of certain response elements in *Sod4A* but not *Sod4* suggests differential functions for the two genes. The 5′ leader sequence of both *Sod4* and *Sod4A* contain a stretch of 5 G's 16 bp before the splice junction of the intron interrupting the leader sequence and 26 bp upstream of the translational start when the intron is removed. This group of G's may have some effect on expression of the genes.

Comparison to other Cu/Zn *Sod* genes: Comparison of *Sod4* and *Sod4A* with other cytosolic Cu/Zn *Sod* genes reveals some interesting features (Figure 4). The Cu/Zn *Sod* sequence from rice (SAKAMOTO *et al.* 1992) contains the identical exon/intron placement as *Sod4* and *Sod4A*. Both the maize and rice cytosolic Cu/Zn *Sod* genes contain an intron interrupting the 5′ untranslated sequence. This indicates very strong conservation of this structure among plants. The exon/intron pattern of human (LEVANON *et al.* 1985) and rat (KIM *et al.* 1993) Cu/Zn *Sod* genes are almost identical to each other. The cytosolic Cu/Zn *Sod* from *Drosophila* (SETO *et al.* 1987) contains only one intron while that of *Neurospora* (CHARY *et al.* 1990) contains three. Yeast Cu/Zn *Sod* has no introns (BERMINGHAM-MCDONOUGH *et al.* 1988). The only intron which appears to be located in a similar position among all of these organisms is the second intron of plants and the first intron of animals. With the exception of *Sod4* and *Sod4A* of maize, computer analysis of the plant cytosolic Cu/Zn *Sod* intron sequences did not reveal any areas of similarity.

Tissue distribution and response to stressors: The cytosolic Cu/Zn *Sod4* and *Sod4A* transcripts are found in most tissues of the maize plant. Previous zymogram analysis indicated that SOD-4 was found in most maize tissues (BAUM and SCANDALIOS 1979). RNA blot analyses using the GSS of *Sod4* and *Sod4A* have indicated that there are some tissues where transcripts of the two genes are more abundant (*i.e.,* 4 dpi scutella, silks, 11 dpp husks) while in other tissues (*i.e.,* leaves, cobs, young and mature tassels) they are less abundant (CAN-

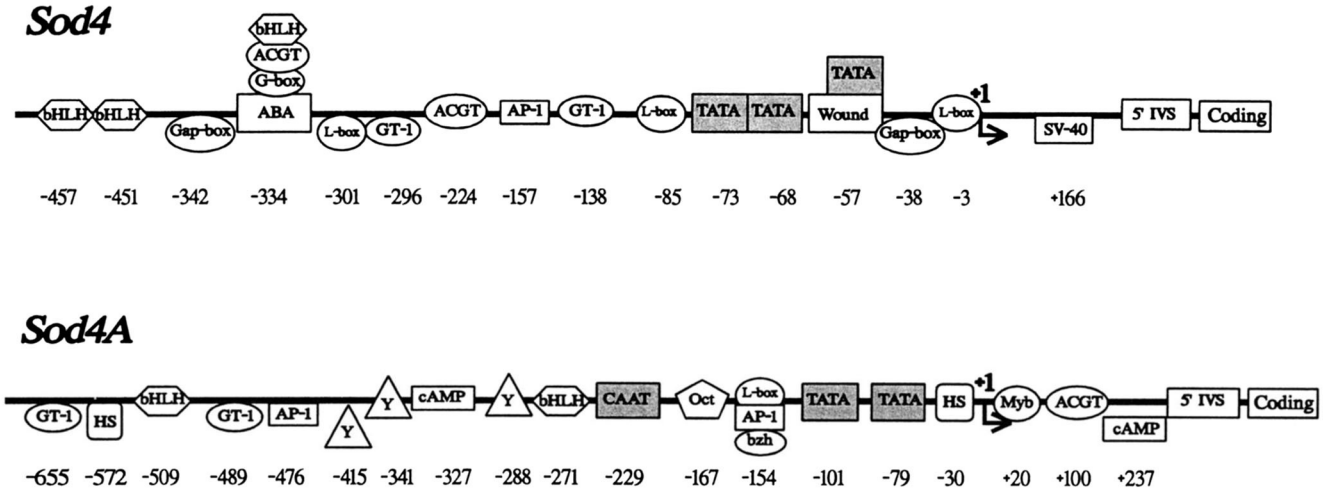

FIGURE 3.—Location of motifs in the promoter regions of the *Sod4* and *Sod4A* genes. The transcription start sites of *Sod4A* and *Sod4* are indicated by +1. Relative positions of the motifs are indicated by the numbers below the line. Motifs straddling the line are found on the sense strand while those below the line are on the antisense strand. Some of the motifs are found in the same position on both the sense and antisense strands (*e.g.*, ACGT and bHLH).

NON and SCANDALIOS 1989; ACEVEDO and SCANDALIOS 1990, 1992).

Plants exposed to elevated temperatures often exhibit changes in metabolic processes as a result of increased oxidative stress. To determine if any changes in *Sod4* and *Sod4A* transcripts occur in response to elevated temperatures, scutellar RNA was isolated from W64A 1–10 dpi seed germinated and grown at 25, 35, and 40°. Duplicate RNA blots were prepared and probed with *Sod4* and *Sod4A* GSS (Figure 5). In scutella of seed germinated at the "normal" 25°, the mRNA levels for the two genes accumulate at different rates. *Sod4* mRNA levels increase greatly at 3 dpi and stay steady until 7 dpi when they begin to decrease slightly to levels similar to 1 and 2 dpi. There is very little *Sod4A* transcript at 1 dpi but then it increases and remains steady at 5–10 dpi. The two genes however, exhibit the same expression pattern (mRNA levels) at 35° and 40°. At 35° there is little *Sod4* or *Sod4A* mRNA at 1 dpi but mRNA levels of both genes increase greatly at 2 dpi and remain steady through 10 dpi at levels similar to those at 25°. At 40° *Sod4* and *Sod4A* transcript levels are very low at 1–3 dpi but increase to a level similar to that of 25° at 4–5 dpi.

TABLE 1

Characteristics of the *Sod4* and *Sod4A* introns

Intron	Placement (aa)[a]	Length (bp)		Percent similarity (%)
		Sod4	*Sod4A*	
1	5′ untranslated region	1436	1238	47
2	26	79	293	39[b]
3	60	105	94	81
4	92	184	185	89
5	102/103	77	76	88
6	129	—[c]	621	—[d]
7	147	—[c]	99	—[d]

[a] aa, amino acid.

[b] The *Sod4A* intron 2 is over three times larger than the *Sod4* intron 2 and the percent similarity is very low. The 5′ and 3′ regions of intron 2 that match have a percent similarity of 71%.

[c] The partial *Sod4* gene does not contain the sixth and seventh intron.

[d] The percent homology could not be calculated for the sixth and seventh introns since the *Sod4* partial gene lacks those two introns.

Ethephon (2-chloroethylphosphonic acid) is often used to study the effects of the phytohormone ethylene on plants which degrade ethephon into ethylene and phosphoric acid. Exposure to ethylene can cause increased respiration and prematuration and early senescence (ABELES 1973). Twelve-day postimbibition W64A seedlings were treated hydroponically for 17 hours with ethephon or phosphoric acid. In leaves isolated from ethephon-treated seedlings, only a slight induction of *Sod4* transcript is detected (Figure 6). No induction of either gene was observed in roots (data not shown). However in stem tissue, there is a significant difference in the level of the two transcripts. While no induction of mRNA levels was detected with *Sod4A*, *Sod4* mRNA levels increased at the 25 mM concentration of ethephon and remained steady through 200 mM concentration, indicating a differential response and regulation of the two genes. No change in mRNA levels were seen in leaf, stem or root tissue from seedlings treated with phosphoric acid (data not shown).

The herbicide paraquat (methyl viologen; 1,1′-dimethyl-4,4′-bipyridinium chloride) acts by accepting an electron from Photosystem I to become a reduced free radical which is then immediately reoxidized by transferring an electron to oxygen forming superoxide (FARRINGTON *et al.* 1973). Paraquat is highly toxic in the light but can also cause damage in the dark by accepting electrons from other sources such as the mitochondrial

TABLE 2

Regulatory motifs found in the *Sod4* and *Sod4A* promoters

Gene	Motif	Sequence[a]	Response (signal/factor)	Literature cited
Sod4	ABA	TCACGTGGTTGTT	ABA regulation	MARCOTTE *et al.* (1989)
Sod4/Sod4A	ACGT	ACGT	Leucine zipper factors	TABATA *et al.* (1989); ARMSTRONG *et al.* (1992)
Sod4/Sod4A	AP-1	TGAGTCA	PMA, UV, hydrogen peroxide	MEYER *et al.* (1993)
Sod4/Sod4A	bHLH	CANNTG	Basic helix-loop-helix proteins	PABO and SAUER (1992)
Sod4A	bzip-hexamer	TGACGY	Leucine zipper factors	KATAGIRI *et al.* (1989)
Sod4A	cAMP-receptor	TGACGTTT	Receptor	MONTMINY *et al.* (1986)
Sod4	G-box	CACGTG	Light	GIULIANO *et al.* (1988)
Sod4	Gap-box	ATGAARA	Light	CONLEY *et al.* (1994)
Sod4/Sod4A	GT-1	KWGTGRWAAWRW	Light	GREEN *et al.* (1988); TERZAGHI and CASHMORE (1995)
Sod4A	Heat Shock (I)	TAAAGGG	Heat Shock	PELHAM (1982)
Sod4A	Heat Shock (II)	CAANNTTC	Heat shock	SORGER (1991)
Sod4/Sod4A	l-box	GATAA	Light	TERZAGHI and CASHMORE (1995)
Sod4A	Myb binding	YAACKG	Reg. of phenylpropanoid prod.	SABLOWSKI *et al.* (1994)
Sod4A	Octamer	ATTTGCAT	Enhancer, activator	HARVEY *et al.* (1982)
Sod4	SV-40	TGTGGWWWG	Enhancer	HATZOPOULOS *et al.* (1988)
Sod4	Wound	TKGTTGAAATAWA	Wounding	PABO and SAUER (1992)
Sod4A	Y-box	GATTGG	Cold stress response, enhancer	WOLFFE (1994)

[a] IUPAC codes for DNA: B = not A; N = any; S = C or G; D = not C; R = A or G; K = G or T; H = not G; Y = C or T; M = A or C; V = not T; W = A or T.

transport chain. It was previously shown in maize that the chloroplastic, cytosolic and mitochondrial SOD proteins increased in response to treatment with paraquat (MATTERS and SCANDALIOS 1986a). However, at the time those studies were performed the cDNA clones had not been isolated. To determine the effect on transcript levels, maize seedlings were treated with increasing concentrations of paraquat in the light and dark. RNA was isolated from the leaves, stems and roots. Subsequent RNA blots with the *Sod4* and *Sod4A* GSS showed a difference in expression of the two genes (Figure 7). In leaves and stems *Sod4* seems to be induced in the dark at the highest concentration of paraquat (0.01 M) and then decreases slightly. *Sod4A* is inhibited by the highest concentration but is induced at lower concentrations (0.001 M) in light and dark grown stems and leaves.

Hydrogen peroxide (H_2O_2) is a product of the dismutation reaction of superoxide dismutase and is removed by catalases and peroxidases. However, if it is inefficiently removed or an excess is present, it can form more superoxide and hydroxyl radicals. Maize scutella from 21 and 28 dpp were placed on MS plates containing various concentrations of H_2O_2 from 0 to 0.1%. RNA blot analysis of 21 dpp embryos shows an increase in transcript levels of both genes up to a peak at 0.1% (Figure 8). At 28 dpp, the mRNA levels of both genes peak at 0.03–0.04% and then decrease slightly at 0.05 and 0.1%.

Seedlings and isolated embryos were treated with other agents which can cause oxidative stress to determine if *Sod4* and *Sod4A* were affected. RNA was isolated

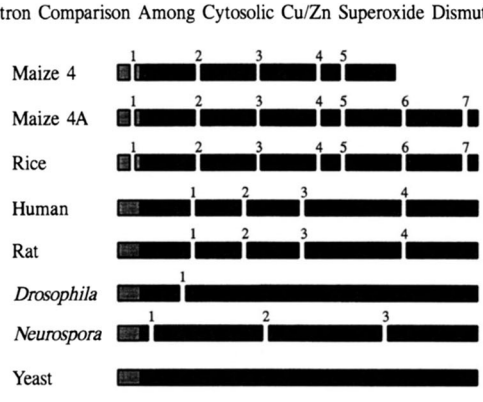

FIGURE 4.—Exon/intron comparison of cytosolic Cu/Zn *Sod* genes. The second intron of the plants and the first intron of human, rat, and Drosophila are located within a span of three to four amino acids. The plant cytosolic Cu/Zn *Sods* have identical number and placement of introns as well as an intron in the 5′ untranslated region.

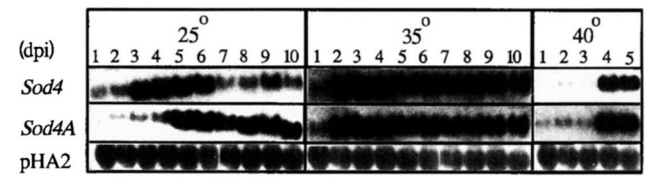

FIGURE 5.—RNA blot analysis of *Sod4* and *Sod4A* in developing scutella from seed germinated and grown for 1–10 days post imbibition (dpi) at 25, 35, and 40°. Duplicate total RNA samples (20 μg) were electrophoresed through a 1.2% agarose-formaldehyde gel, transferred to nitrocellulose and probed with the *Sod4* or *Sod4A* GSS. The pea ribosomal DNA probe (pHA2) was used as a loading control for all RNA blots.

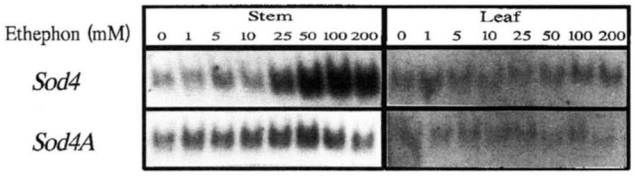

FIGURE 6.—RNA blot analysis of *Sod4* and *Sod4A* mRNA levels in tissues of seedlings treated with increasing concentrations of ethephon. Twelve-day seedlings were exposed to ethephon through hydroponic uptake for 17 hr. Duplicate RNA blots with 20 μg total RNA per lane from leaves and stems were probed with *Sod4* and *Sod4A* GSS. In stem tissue, the *Sod4* transcript increases in response to ethephon while the *Sod4A* transcript does not.

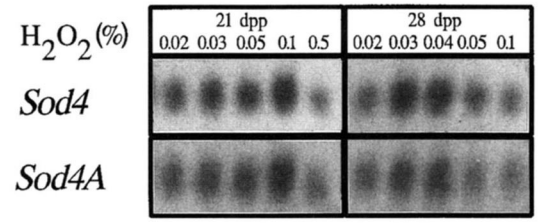

FIGURE 8.—RNA blot analysis of *Sod4* and *Sod4A* mRNA levels in 21 and 28 days postpollination (dpp) scutella treated with increasing concentrations of hydrogen peroxide. Duplicate total RNA samples (20 μg) were electrophoresed through 1.2% agarose-formaldehyde gel, transferred to nitrocellulose and probed with the *Sod4* or *Sod4A* GSS.

from leaves exposed to various light regimens (12/12 hr light/dark, constant light, constant dark) and different wavelengths of the light spectrum (white, blue, red, far red, red/far red). RNA was also isolated from embryos treated with salicylic acid. Subsequent RNA blots however, showed no changes in transcript levels for *Sod4* or *Sod4A* under these conditions. However, treatment of embryos with the light-activated fungal toxin cercosporin resulted in an initial decrease followed by a dose-dependent increase of both *Sod4* and *Sod4A* transcripts (Figure 9) (WILLIAMSON and SCANDALIOS 1992). Further experiments involving other stressors or signals may help to further define differences and similarities in the regulation and functional roles of these two closely related genes.

DISCUSSION

The maize *Sod4* and *Sod4A* genes show a remarkable similarity in their structure. A previous paper (CANNON and SCANDALIOS 1989) discussed the two cDNAs in detail and presented evidence that the two genes encode two biochemically indistinct proteins. There are two amino acid changes between the SOD-4 and SOD-4A proteins at positions 12 and 29. The purpose of maize having two such closely related *Sod* genes may be a consequence of selection pressure to provide maximal antioxidant defenses under constantly changing environments. RNA data presented in this paper indicate that there is a differential tissue-specific response between *Sod4* and *Sod4A* to certain stress signals. It is also possible that the *Sod4* and *Sod4A* genes may be located in different regions of the cytosol and this is currently being investigated using a tissue printing technique (YE *et al.* 1992).

The promoters of *Sod4* and *Sod4A* contain some of the same transcription regulatory elements or enhancers, but also contain different motifs which may help to explain the differential regulation of these two almost identical genes. The *Sod4A* promoter sequence contains several CAAT and TATA boxes while only TATA boxes are present in the *Sod4* promoter although almost all are found farther upstream than usual and may not be functionally significant. The ABA response element is located only in the *Sod4* promoter and treatment of maize embryos with ABA causes an increase in *Sod4* mRNA levels, but not in those of *Sod4A*. Several known light regulatory elements are found in the *Sod4* and *Sod4A* promoters. However, preliminary RNA results have indicated different light regimens, or various wavelengths of the light spectrum do not have any detectable effects on the expression of either *Sod4* or *Sod4A*. Further studies may help to reveal those elements that are functional.

The exon/intron patterns of *Sod4* and *Sod4A* are identical. Although *Sod4* is only a partial clone, data from *Sod4A* as well as the exon/intron location of other plant cytosolic Cu/Zn *Sod* genes deem it likely that placement of the two missing introns of *Sod4* will be the same as *Sod4A*. The DNA sequence similarity between

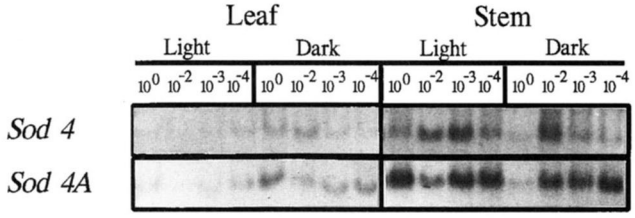

FIGURE 7.—RNA blot analysis of *Sod4* and *Sod4A* mRNA levels in leaf and stem tissue isolated from seedlings treated with paraquat in the light and dark. Twelve-day seedlings were exposed to paraquat through hydroponic uptake for 17 hr. Duplicate RNA blots with 20 μg total RNA per lane from leaves and stems were probed with *Sod4* or *Sod4A* GSS.

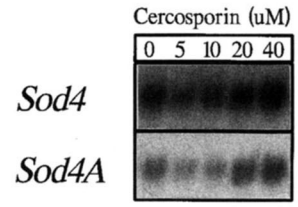

FIGURE 9.—RNA blot analysis of *Sod4* and *Sod4A* mRNA levels in post pollination embryos treated with the light-activated fungal toxin cercosporin. Duplicate RNA blots were prepared with 20 μg total RNA per lane and hybridized with the *Sod4* or *Sod4A* GSS. Hybridization with the fragment of pea ribosomal DNA did not show any loading variances.

corresponding introns of *Sod4* and *Sod4A* is also very high. This is not really surprising considering the high homology of the two cDNAs. The AT content of the *Sod4* and *Sod4A* introns ranges from a high of 72.8% for intron 2 of *Sod4A* to a low of 50.5% for intron 6 of *Sod4A*. The average for all seven introns is 62% for *Sod4* and *Sod4A*. It has been proposed that splicing of the RNA precursor in monocots and dicots is dependent on AT content (GOODALL and FILIPOWICZ 1991). Processing of various plant introns in tobacco and maize protoplasts revealed that monocots processed all introns (both monocot and dicot) efficiently while in tobacco only the introns with high AT content spliced efficiently (GOODALL *et al.* 1992).

To date, full-length or nearly full-length genomic cytosolic Cu/Zn *Sod* sequences have been reported from humans (LEVANON *et al.* 1985), rat (KIM *et al.* 1993), *Drosophila* (SETO *et al.* 1987), *Neurospora* (CHARY *et al.* 1990), yeast (BERMINGHAM-MCDONOUGH *et al.* 1988), and rice (SAKAMOTO *et al.* 1992). The position of introns in the published *Sod* genomic sequences (Fig. 4) indicates that all of the plant Cu/Zn *Sod* introns lie in exactly the same position and each has an intron interrupting the 5′ leader sequence. The vertebrate sequences do not contain the 5′ intron and yeast contains no introns. A recently reported chloroplastic Cu/Zn *Sod* gene from tomato does not contain the 5′ intron and some of its other introns lie in positions different from those of the cytosolic genes (KARDISH *et al.* 1994). A few other genes containing an intron interrupting the 5′ leader sequence have been reported including plant and vertebrate actin genes (PEARSON and MEAGHER 1990), insulin genes (PERLER *et al.* 1980) and the maize *Shrunken-1* gene (CLANCY *et al.* 1994). Interruption of the 5′ leader sequence by an intron is commonly found in mammalian genes. In the actin genes the 5′ leader intron may be conserved because it is found in a similar position in plants and vertebrates while most of the other actin introns are located in different positions. When the intron placement of the plant Cu/Zn *Sod* genes is compared to the human Cu/Zn *Sod,* four of the introns are located in positions similar to the four positions of the human Cu/Zn *Sod;* the remaining three introns occupy different positions. These extra introns in the plant genomic sequences could be ancient sequences in the progenitor *Sod* gene which, after plants and animals diverged, the plants needed but the animals were able to discard, or the extra introns may be the result of a later insertion. The origin of introns is a subject of much debate (GILBERT 1978; PALMER and LOGSDON 1991). One theory proposes that exons, which are considered to contain information for structural and functional portions of a protein, are duplicated to form the final protein structure, "exon shuffling" (GILBERT 1978). In studies of the bovine Cu/Zn SOD, it was proposed (MCLACHAN 1980) that present day forms of Cu/Zn SOD may be the result of a gene duplication because of its structural symmetry. The identical intron positions of the plant Cu/Zn *Sod* genes suggests conservation of these introns, whether due to structural stability or a regulatory role. The intron sequences of the plant cytosolic Cu/Zn *Sods* have been compared and there appears to be little similarity between the species. As noted previously, the comparable introns of *Sod4* and *Sod4A* are highly homologous. Some regions of similarity might be expected in the 5′ intron since this intron is found only in the plant *Sod* genes. Intron sequences may contain regulatory elements and have been shown to have effects on gene expression in plants and animals (BUCHMAN and BERG 1988; CLANCY *et al.* 1994). For example, in *Neurospora* the first intron of the Cu/Zn *Sod* contains sequences similar to those of UAS-1, the transcriptional regulatory site of the yeast *CYC1* gene and to UAS from the Mn *Sod* of yeast (CHARY *et al.* 1990). In the *Sod4* and *Sod4A* genes there is a 4–5 bp "G" stretch in the 5′ leader sequence 16 bp upstream of the intron interrupting the 5′ leader sequence. If the intron is removed, this "G" stretch is located 26 bp upstream of the translational start of all the plant genomic cytosolic Cu/Zn *Sod* sequences. The 26 bp prior to the translational start of these genes are also highly conserved. Whether this sequence has any impact on expression levels of Cu/Zn *Sod* remains to be determined.

Structural analysis of the Cu/Zn SOD protein indicates that exon/intron boundaries generally lie in regions of the protein surface where any change in sequence would not affect the structure or function of the protein (GETZOFF *et al.* 1989). Three of the four introns of the human Cu/Zn *Sod* map to the protein surface. The second and third introns of *Sod4* and *Sod4A* (as well as those of rice and tobacco) are found in positions similar to the human Cu/Zn *Sod* first and second introns. These two introns map to the loop separating the first β-strand and the Zn subloop, respectively. The five remaining introns map to locations different (a separation of at least 10–15 amino acids) from the human *Sod* gene. The fourth maize intron is located on the fifth loop separating two of the β-strands. The fifth intron is found at the beginning of the second Greek key loop. The sixth intron is found on the electrostatic channel loop while the seventh is located at the beginning of a disulfide bridge. It appears that all the introns probably map to the protein surface.

Steady state levels of *Sod4* and *Sod4A* transcripts can be found in almost all maize tissues (CANNON and SCANDALIOS 1989; ACEVEDO and SCANDALIOS 1990, 1992). This corresponds to the appearance of SOD protein in all maize tissues studied (BAUM and SCANDALIOS 1979). Changes in these levels can be induced under some conditions of oxidative stress.

The transcripts of *Sod4* and *Sod4A* increase steadily in the developing maize seedling, in response to the increased demands to remove oxygen radicals formed

during metabolic processes (Figure 5). At 25° there is a difference in transcript accumulation for the two genes. *Sod4* increases gradually and remains steady at 3–6 dpi and then returns to earlier levels while *Sod4A* is barely present at 1 dpi, then increases until 5 dpi and remains steady levels through 10 dpi, indicating possible temporal regulation of the two genes. Seedlings grown at 35° exhibited no difference in transcript levels between the two genes. However, the mRNA levels of the two genes accumulate much earlier (2 dpi) than at 25° although levels appear to be about the same. When the seedlings were grown at 40° there was no difference in the mRNA levels between the two genes. Earlier results of SOD protein levels in line W64A grown at 25, 35, and 40°, showed no significant differences in total SOD activity levels (MATTERS and SCANDALIOS 1986b).

When developing seedlings are treated with agents which cause oxidative stress, a differential, tissue-specific response of the two closely related *Sod4* and *Sod4A* genes occurs under some conditions. This response is most notable when seedlings are exposed to ethephon which is reduced to ethylene and phosphoric acid in the plant. No adverse effects are seen on the seedlings at 0–10 mM concentrations of ethephon. Beginning with 25 mM ethephon the seedling stems appear reddish purple due to anthocyanin production and the leaves begin to wilt. At 200 mM the leaves are mostly brown. *Sod4* transcript levels increase dramatically in stem and leaf tissue while those of *Sod4A* remain steady (Figure 6). Neither gene contains an ethylene-binding response element in the promoter region. There is no difference in mRNA levels between the two genes in root tissue. No change in mRNA levels of leaf, stem, and root tissue was seen when the seedlings were treated with phosphoric acid, indicative that the response is most likely due to ethylene. The two genes also show a difference in responsiveness to the herbicide paraquat. Treatment of seedlings with paraquat results in opposite expression of the two genes (Figure 7). The *Sod4* transcript is induced at higher levels while *Sod4A* is inhibited by those conditions. However, treatment with other chemicals does not always result in a differential response between the two genes although they are induced (hydrogen peroxide). Still other treatments have no effect on the two genes at all (salicylic acid, elevated temperature). Further experiments with other agents which can cause oxidative stress may help to explain the mechanisms leading to the differential response of the two genes.

In summary, characterization of the maize cytosolic Cu/Zn *Sod4* and *Sod4A* has revealed two genes with almost identical structure and DNA sequence which are expressed differently under certain conditions of oxidative stress. These findings suggest that even though these two genes are very similar structurally, they may have evolved some unique functional roles to aid the organism in its capacity to cope with constantly changing adverse oxidative environmental insults.

We thank Drs. LINGQIANG GUAN, ALEXIOS POLIDOROS and PHOTINI MYLONA for constructive comments on the manuscript. This work was supported by Research Grant (R-819360) from the U.S. Environmental Protection Agency (Environmental Biology Program) to J.G.S.

LITERATURE CITED

ABELES, F. B., 1973 *Ethylene in Plant Biology*. Academic Press, New York.

ACEVEDO, A., and J. G. SCANDALIOS, 1990 Expression of the catalase and superoxide dismutase genes in mature pollen in maize. Theor. Appl. Genet. **80:** 705–711.

ACEVEDO, A., and J. G. SCANDALIOS, 1992 Differential expression of the catalase and superoxide dismutase genes in maize ear shoot tissues. Plant Cell Physiol. **33:** 1079–1088.

ARMSTRONG, G. A., B. WEISSHAAR and K. HAHLBROCK, 1992 Homodimeric and heterodimeric leucine zipper proteins and nuclear factors from parsley recognize diverse promoter elements with ACGT cores. Plant Cell **4:** 525–537.

BAUM, J. A., and J. G. SCANDALIOS, 1979 Developmental expression and intracellular localization of superoxide dismutases in maize. Differentiation **13:** 133–140.

BEACHY, R. N., Z.-L. CHEN, R. B. HORSCH, S. G. ROGERS, N. L. HOFFMAN *et al.*, 1985 Accumulation and assembly of soybean β-conglycinin in seeds of transformed petunia plants. EMBO J. **4:** 3047–3053.

BERMINGHAM-MCDONOGH, O., E. B. GRALLA and J. S. VALENTINE, 1988 The copper, zinc-superoxide dismutase gene of *Saccharomyces cerevisiae:* cloning, sequencing, and biological activity. Proc. Natl. Acad. Sci. USA **85:** 4789–4793.

BIRNBOIM, H. C., and J. DOLY, 1979 A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:** 1513–1523.

BREATHNACH, R., and P. CHAMBON, 1981 Organization and expression of eucaryotic split genes coding for proteins. Annu. Rev. Biochem. **50:** 349–383.

BUCHMAN, A. R., and P. BERG, 1988 Comparison of intron-dependent and intron-independent gene expression. Mol. Cell. Biol. **8:** 4395–4405.

CANNON, R. E., J. A. WHITE and J. G. SCANDALIOS, 1987 Cloning of a cDNA for maize superoxide dismutase 2 (SOD2). Proc. Natl. Acad. Sci. USA **84:** 179–193.

CANNON, R. E., and J. G. SCANDALIOS, 1989 Two cDNAs encode two nearly identical Cu/Zn superoxide dismutase proteins in maize. Mol. Gen. Genet. **219:** 1–8.

CHARY, P., R. A. HALLEWELL and D. O. NATVIG, 1990 Structure, exon pattern and chromosome mapping of the gene for cytosolic copper-zinc Sod (Sod-1) from *Neurospora crassa*. J. Biol. Chem. **265:** 18961–18967.

CLANCY, M., V. VASIL, L. C. HANNAH and I. K. VASIL, 1994 Maize *Shrunken-1* intron and exon regions increase gene expression in maize protoplasts. Plant Sci. **98:** 151–161.

CONLEY, T. R., S. C. PARK, H. B. KWON, H. P. PENH and M. C. SHIH, 1994 Characterization of cis-acting elements in light regulation of the nuclear gene encoding the A subunit of chloroplast isozymes of glyceraldehyde 3-phosphate dehydrogenase from *Arabidopsis thaliana*. Mol. Cell. Biol. **14:** 2523–2533.

FARRINGTON, J. A., M. EBERT, E. J. LAND and K. FLETCHER, 1973 Bipyridylium quaternary salts and related compounds: v. Pulse radiolysis studies of the reaction of paraquat radical with oxygen. Implications for the mode of action of bipyridyl herbicides. Biochim. Biophys. Acta **314:** 372–381.

FEINBERG, A. P., and B. VOGELSTEIN, 1983 A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:** 6–13, 266–267.

FRIDOVICH, I., 1978 The biology of oxygen radicals. Science **201:** 875–880.

FRIDOVICH, I., 1995 Superoxide radical and superoxide dismutases. Annu. Rev. Biochem. **64:** 97–112.

GETZOFF, E. D., J. A. TAINER, M. M. STEMPIEN, G. I. BELL and R. A. HALLEWELL, 1989 Evolution of CuZn superoxide dismutase and

the greek key *B*-barrel structural motif proteins. Proteins **5:** 322–336.

GILBERT, W., 1978 Why genes in pieces? Nature **271:** 501.

GIULIANO, G., E. PICHERSKY, V. S. MALIK, M. P. TIMKO, P. A. SCOLNIK *et al.*, 1988 An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. Proc. Natl. Acad. Sci. USA **85:** 7089–7093.

GOODALL, G. J., and W. FILIPOWICZ, 1991 Different effects of intron nucleotide composition and secondary structure on pre-mRNA splicing in monocot and dicot plants. EMBO J. **10:** 2635–2644.

GOODALL, G. J., T. KISS and W. FILIPOWICZ, 1992 Nuclear RNA splicing and small nuclear RNAs and their genes in higher plants, pp. 255–296 in *Oxford Surveys of Plant Molecular and Cell Biology*, Vol. 7, edited by B. J. MIFLIN. Oxford University Press, Oxford.

GREEN, P. J., M. H. YONG, M. CUOZZO, Y. KANO-MURAKAMI, P. SILVERSTEIN *et al.*, 1988 Binding site requirements for pea nuclear protein factor GT-1 correlate with sequences required for light-dependent transcriptional activation. EMBO J. **7:** 4035–4044.

GUILTINAN, M. J., W. R. MARCOTTE and R. S. QUATRANO, 1990 A plant leucine zipper protein that recognizes an abscissic acid response element. Science **250:** 267–271.

HARVEY, R. P., A. J. ROBINS and J. R. E. WELLS, 1982 Independently evolving chicken histone H2B genes: identification of a ubiquitous H2B-specific 5′ element. Nucleic Acids Res. **10:** 7851–7864.

HATZOPOULOS, A. K., U. SCHLOKAT and P. GRUSS, 1988 Enhancers and other cis-acting regulatory elements, pp. 43–96 in *Transcription and Splicing*, edited by B. D. HAMES and D. M. GLOVER. IRL Press, Washington, DC.

JORGENSEN, R. A., R. E. CUELLAR, W. F. THOMPSON and T. A. KAVANAUGH, 1987 Structure and variation in ribosomal RNA genes of pea: characterization of a cloned rDNA repeat and chromosomal rDNA variants. Plant Mol. Biol. **8:** 3–12.

KANEMATSU, S., and K. ASADA, 1990 Characteristic amino acid sequences of chloroplast and cytosol isozymes of Cu/Zn superoxide dismutase in spinach, rice and horsetail. Plant Cell Physiol. **31:** 99–112.

KARDISH, N., N. MAGAL, D. AVIV and E. GALUN, 1994 The tomato gene for the chloroplastic Cu,Zn superoxide dismutase: regulation of expression imposed in transgenic tobacco plants by a short promoter. Plant Mol. Biol. **25:** 887–897.

KATAGIRI, F., E. LAM and N. H. CHUA, 1989 Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. Nature **340:** 727–730.

KERNODLE, S. P., R. E. CANNON and J. G. SCANDALIOS, 1993 Rapid and simple phage DNA isolation. Biotechniques **14:** 360.

KIM, Y. A., H. Y. YOO, G. JUNG, J.-Y. KIM and H. M. RHO, 1993 Isolation and analysis of the rat genomic sequence encoding Cu/Zn superoxide dismutase. Gene **133:** 267–271.

LEVANON, D., J. LIEMAN-HURWITZ, N. DAFNI, M. WIGDERSON, L. SHERMAN *et al.*, 1985 Architecture and anatomy of the chromosomal locus in human chromosome 21 encoding the Cu/Zn superoxide dismutase. EMBO J. **4:** 77–84.

MANIATIS, T., E. F. FRITSCH and T. SAMBROOK, 1982 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

MARCOTTE, W. R., S. H. RUSSELL and R. S. QUATRANO, 1989 Abscissic acid-responsive sequences from the *Em* gene of wheat. *Plant Cell* **1:** 969–976.

MATTERS, G. L., and J. G. SCANDALIOS, 1986a Effect of the free radical-generating herbicide paraquat on the expression of the superoxide dismutase (*Sod*) genes in maize. Biochim. Biophys. Acta **882:** 29–38.

MATTERS, G. L., and J. G. SCANDALIOS, 1986b Effect of elevated temperature on catalase and superoxide dismutase during maize development. Differentiation **30:** 190–196.

MCLACHAN, A. D., 1980 Repeated folding pattern in copper-zinc superoxide dismutase. Nature **285:** 267–270.

MEYER, M., R. SCHRECK, and P. A. BAEURLE, 1993 H_2O_2 and antioxidants have opposite effects on activation of NH-kB and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. EMBO J. **12:** 2005–2015.

MONTMINY, M. R., K. A. SEVARINO, J. A. WAGNER, G. MANDEL and R. H. GOODMAN, 1986 Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc. Natl. Acad. Sci. USA **83:** 6682–6686.

OHME-TAKAGI, M., and H. SHINSHI, 1995 Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. Plant Cell **7:** 173–182.

PABO, C. O., and R. T. SAUER, 1992 Transcription factors: structural families and principles of DNA recognition. Annu. Rev. Biochem. **50:** 349–383.

PALMER, J. D., and J. M. LOGSDON, JR., 1991 The recent origins of introns. Curr. Opin. Genet. Dev. **1:** 470–477.

PEARSON, L., and R. B. MEAGHER, 1990 Diverse soybean actin transcripts contain a large intron in the 5′ untranslated leader: structural similarity to vertebrate muscle actin genes. Plant Mol. Biol. **14:** 513–526.

PELHAM, H. R. B., 1982 A regulatory upstream promoter element in the *Drosophila* Hsp70 heat shock gene. Cell **30:** 517–528.

PERLER, F., A. EFSTRATIADIS, P. LOMEDICO, W. GILBERT, R. KOLODNER *et al.*, 1980 The evolution of genes: the chicken preproinsulin gene. Cell **20:** 555–666.

RUSHMORE, T. H., M. R. MORTON and C. B. PICKETT, 1991 The antioxidant responsive element: activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J. Biol. Chem. **266:** 11632–11639.

SABLOWSKI, R. W. M., E. MOYANO, F. A. CULIANEZ-MACIA, W. SCHUCH, C. MARTIN *et al.*, 1994 A flower-specific Myb protein activated transcription of phenylpropanoid biosynthetic genes. EMBO J. **13:** 128–137.

SAKAMOTO, A., T. OKUMURA, H. OHSUGA, and K. TANAKA, 1992 Genomic structure of the gene for copper/zinc superoxide dismutase in rice. FEBS Lett. **301:** 185–189.

SANGER, F., S. NICKLEN, and A. R. COULSON, 1977 DNA sequencing with chain-terminating inhibitor. Proc. Natl. Acad. Sci. USA **74:** 5463–5467.

SCANDALIOS, J. G., 1993 Oxygen stress and superoxide dismutases. Plant Physiol. **101:** 7–12.

SETO, N. O. L., S. HAYASHI and G. M. TENER, 1987 The sequence of the Cu/Zn superoxide dismutase gene of *Drosophila*. Nucleic Acids Res. **15:** 10601.

SORGER, P. K., 1991 Heat shock factor and the heat shock response. Cell **65:** 363–366.

TABATA, T., H. TAKASE, S. TAKAYAMA, K. MIKAMI, T. NAKAYAMA *et al.*, 1989 A protein that binds to a cis-acting element of wheat histone genes has a leucine zipper motif. Science **245:** 965–967.

TERZAGHI, W. B., and A. R. CASHMORE, 1995 Light regulated transcription. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46:** 445–474.

WILLIAMSON, J. D., and J. G. SCANDALIOS, 1992 Differential responses of maize catalase and superoxide dismutases to the photoactivated fungal toxin cercosporin. Plant J. **2:** 351–358.

WOLFFE, A. P., 1994 Structural and functional properties of the evolutionarily ancient Y-box family of nucleic acid binding proteins. BioEssays **16:** 245–251.

YE, Z-H., Y.-R., SONG and J. E. VARNER, 1992 Gene expression in plants, pp. 95–123 in *Tissue Printing*, edited by P. D. REID and R. F. PONT-LEZICA. Academic Press, Inc., San Diego.

ZHU, D., and J. G. SCANDALIOS, 1993 The maize mitochondrial manganese superoxide dismutases (MnSOD's) are a differentially expressed multigene family. Proc. Natl. Acad. Sci. USA **90:** 9310–9314.

Communicating editor: S. L. ALLEN