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Differential Expression of Two SOD (Superoxide Dismutase) Genes from Small Radish (*Rhaphanus sativus* L. var. *sativus*)

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We have isolated two superoxide dismutase cDNA clones (*RsCu/ZnSod* and *RsFeSod*) from small radish (*Raphanus sativus* L.) by cDNA library screening. *RsCu/ZnSod* is 563 bp long, with an open reading frame of 153 amino acids, and corresponds to a protein of predicted molecular mass 15.1 kDa and a pI of 5.44. The 823-bp *RsFeSod* has an ORF of 213 amino acids, corresponding to a protein of predicted molecular mass 25.4 kDa and a pI of 8.77. Their nucleotide and deduced amino acid sequences show the highest homology with those of *Arabidopsis*. Genomic Southern blot analysis, using each cDNA clone as probe, has revealed that the SOD genes are present as at least two copies in the small radish genome. Nondenaturing polyacrylamide gels for SOD activity has demonstrated the presence of several isozymes, depending on the organ type and developmental stage. These *RsSod* genes also have differential expression patterns in response to treatments with white light, xenobiotics, UV, osmoticums, plant hormones, and salicylic acid. Therefore, we suggest that they are involved in an antioxidative defense mechanism against stress induced by environmental change.

Keywords: light, osmoticum, *Raphanus*, small radish, SOD, xenobiotics

Superoxide dismutase (SOD; EC 1.15.1.1) plays a central role in protecting plants against oxidative stress. As the key enzyme, it catalyzes the dismutation of the superoxide radical into hydrogen peroxide and molecular oxygen ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$), which are eventually removed by catalase and peroxidase (Bannister et al., 1987; Scandalios, 2005). Three types of SOD are classified based on the metal co-factor present at the catalytic site. These include copper/zinc (Cu/ZnSOD), iron (FeSOD), or manganese (MnSOD) SODs, which also can be identified by their differential sensitivity to KCN and hydrogen peroxide (Scandalios, 1997). Cu/ZnSOD is usually found in the cytosol (or chloroplasts) of eukaryotic cells, and is characterized as sensitive to both KCN and H_2O_2. FeSOD, present in some higher plant chloroplasts, is sensitive only to H_2O_2 while MnSOD exists in prokaryotes and the mitochondria, and is insensitive to both inhibitors. The location of SOD in these various subcellular compartments is thought to be associated with ensuring the efficient scavenging of superoxide radicals at their sites of formation (Bowler et al., 1992, 1994). However, the functioning of each SOD in response to plant oxidative stress is still unclear and sometimes contradictory (Scandalios, 2005).

Unlike most other organisms that have only one of each type of SOD in their cellular compartments, plants contain multiple SOD isozymes encoded by more than one gene. Examples include the nine isoenzymes in maize (Baum and Scandalios, 1981) and the seven in *Arabidopsis* (Kliebenstein et al., 1998). The sequences of many SOD genes have been reported in maize, tobacco, tomato, rice, hot pepper, cassava, and liverwort (Perl-Treves et al., 1988; Bowler et al., 1989; Cannon and Scandalios, 1989; Kaminaka et al., 1997; Tanaka et al., 1998; Kwon and An, 1999; Lee et al., 1999). Likewise, the role of SODs under environmental stresses has been studied extensively (Bowler et al., 1992; Scandalios, 1997; Sakaguchi et al., 2004). Tolerance to oxidative, drought, salt, and cold stresses is reported to increase in transgenic plants that over-produce these SOD genes (van Camp et al., 1996; van Breusegem et al., 1999; Wang et al., 2004, 2005).

Cu/ZnSODs are found throughout the plant cell, existing in both chloroplastic and cytosolic forms. The deduced amino acid sequences of these two isoforms show approximately 70% similarity, whereas the similarity is about 90% among the chloroplastic Cu/ZnSOD and 80 to 90% among the cytosolic Cu/ZnSOD. In several species, regulation of the cytosolic Cu/ZnSOD depends upon the developmental stage (Acevedo and Scandalios, 1991); its expression is also induced by chemical treatment or environmental stresses such as paraquat, heat-shock, chilling, mechanical wounding, ozone, salt, hormones, and light exposure (Perl-Teves and Galun, 1991; Sakamoto et al., 1992; Guan and Scandalios, 1998).

FeSOD has not been found in animals or fungi, but is present in a limited number of seed plants, e.g., *Arabidopsis thaliana*, tobacco (van Camp et al., 1990), soybean (Crowell and Amasino, 1991b), and rice (Kaminaka et al., 1999). Its absence in animal species has led researchers to propose that the FeSOD gene originated in the plastid before moving to the nuclear genome. Nonetheless, many seed plants, including maize, exhibit no FeSOD activity. However, such activity and transcript of the FeSOD gene has been detected in response to various stimuli and at certain developmental stages in barley (Casano et al., 1994), tobacco (Tsang et al., 1991; Kurepa et al., 1997), and rice (Kaminaka et al., 1999).

MnSOD is widely distributed in prokaryotic and eukaryotic organisms, most often being found in the mitochondrial matrix. This suggests a correlation between its expression and mitochondrial respiratory activity. MnSOD cDNA clones have been isolated from maize, rice, tobacco, *Arabidopsis*, and pea (White and Scandalios, 1988; Bowler et al., 1989; Sakamoto et al., 1993) and have revealed a

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highly conserved sequence based on the striking amino acid homology between phylogenetically distant organisms (Alscher et al., 2002). In contrast to the general inducibility of cytosolic SOD, mitochondrial MnSOD shows only minimal induction in response to various environmental stresses (Tsang et al., 1991; Kliebenstein et al., 1998; Mylona et al., 1998).

Radish (*Rhaphanus sativus*), which belongs to the same Brassicaceae family as *Arabidopsis*, is a well-known model plant system, and a very useful vegetable crop. To elucidate the molecular mechanisms of antioxidant enzymes in the small radish, we previously isolated and characterized the *RsMnSod* gene (Kwon and An, 2003) plus three different catalase genes (unpublished data). Here, we report the isolation of two other SOD cDNAs (*RsCu/ZnSod* and *RsFeSod*) and their expression patterns in different organs, over time, and in response to various environmental (or chemical) treatments.

MATERIALS AND METHODS

Plant Material and Treatments

Seeds of the small radish (*R. sativus* L.) were purchased from TAKII Seed Company (Japan) and stored at 4°C until used. They were germinated in the dark at room temperature for 3 to 4 d, then transferred to soil and reared in a growth chamber at 30°C under a 14-h photoperiod (150 to 160 mol m^{-2} s^{-1}). For our UV and other light experiments, seeds were germinated at 25°C for 3 to 4 d in the dark before the seedlings were transferred to growth chambers and treated with UV (60 μW cm^{-2}) for various periods and in combination with other light sources. For the chemical treatment, seeds were first placed in a dark chamber at 25°C for at least 3 to 4 d to ensure uniform germination before their transfer. When the seedlings were approximately 3 cm tall, they were incubated in a liquid MS medium containing an osmoticum (10% sucrose, 10% mannitol, or 100 mM NaCl), 2 mM salicylic acid (SA), or phytohormones (ABA or IAA) for various time periods. For experiments with xenobiotics, compounds of 50 μM methyl viologen (Sigma, USA), 50 μM plumbagin (Sigma), or 25 μM cercosporin (Sigma) were sprayed on the leaves of young, 12-cm-tall plants that had been grown in soil for two weeks. All tissue samples were frozen immediately in liquid nitrogen and stored at -80°C until they were analyzed as previously described (Kwon and An, 2003).

PCR Reaction for Probe Preparation

cDNA synthesized from seedling mRNA (or genomic DNA) was used as template for PCR. Degenerate oligonucleotide primers (Table 1) corresponding to two conserved regions in the plant SOD genes were used as primers for these reactions. The program included denaturation at 94°C for 5 min; followed by 30 PCR cycles of annealing at 45°C for 1 min, and polymerization at 72°C for 1 min; then a final denaturation at 94°C for 45 s. PCR fragments were cloned into the *Sma*I site of the pUC19 vector.

Enzyme Activity on the Native Gel

To analyze SOD activity, we centrifuged cell-free protein extracts in 50 mM potassium phosphate buffer (pH 7.0) at 13000 rpm and 4°C for 10 min. The protein concentration of the supernatant was measured according to the method of Bradford (1976). SOD isozymes were separated on a 7% nondenaturing polyacrylamide gel at 120 V for 15 h at 4°C, and the gel was then stained for SOD activity (Beauchamp and Fridovich, 1971).

Construction and Screening of a cDNA Library for Small Radish

Total RNA isolation, cDNA library construction, and SOD cDNA clone screening have been described previously (Kwon and An, 2003). Hybridization was carried out by the method of Sambrook et al. (1989). Sequences of the selected clones were analyzed with the BLAST program and the Expasy Molecular Biology Server, and phylogenetic analysis was carried out via the PHYLIP program.

Southern and Northern Analyses

Genomic DNA was prepared from radish seedlings according to the method of Ausubel et al. (1987). Gel blotting and filter hybridization were conducted as described by Kwon and An (2003). Total RNA was extracted from whole seedlings or individual tissues by using the RNA-PLUS extraction solution (Quantum, France) according to the manufacturer's protocol. Northern blots were hybridized with random-primed DNA probes synthesized with the Prime-a-Gene Labeling System (Promega, USA), using gene-specific PCR products of the three SOD cDNA clones, and the 18s rRNA PCR amplified fragments from small radish as templates. Hybridization was carried out at 62°C for 20 h before the filters were washed and visualized by autoradiography at -80°C.

RESULTS AND DISCUSSION

Isolation and Characterization of Cu/Zn- and Fe-SOD cDNA Clones

We previously isolated a cDNA clone of small radish that encodes a mitochondrial MnSOD (Kwon and An, 2003).

Table 1. Primer sequences for the amplification of *RsCu/Zn*Sod, *RsFeSod*, *RsMn*Sod, and 18S rRNA.

1. Primers used for SOD coding region	(bp)
*RsCu/ZnSod*F; cctgg(ac)ct(ct)catgg(gc)tt(ct)cat	
*RsCu/ZnSod*R; ctgag(atg)tc(ag)tgtcc(at)ccctt	285
*RsFeSod*F; ct(ct)cc(at)gc(at)ttcaacaa(tc)gc	
*RsFeSod*R; gta(at)gcatg(tc)tccca(ag)ac(ag)tc	306
*RsMnSod*F; ga(ag)gg(at)ggtgg(gtc)ga(ga)cc(gat)cc	
*RsMnSod*R; gta(at)gcatg(tc)tccca(ag)ac(ag)tc	258
2. Primer used with 18S rRNA	
18S rRNAF; tacctggttgatcctgcc	
18S rRNAR; ccaatggatcctcgttaa	550

(A) *RsCu/ZnSod*

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caggcatttcatcttcattcatttccaaaaggggtaccctgagatca
atg ggc aag gga gtt gca gtg ttg aac agc agc gag
 M   G   K   G   V   A   V   L   N   S   S   E  12
ggt gtt aag gga acc atc ttc ttc acc cag gaa gga
 G   V   K   G   T   I   F   F   T   Q   E   G  24
aac ggt tcc acc act gtg act gga act gtt tct ggc
 N   G   S   T   T   V   T   G   T   V   S   G  36
                                 ★       ★
ctt aag cct ggt ctc cat ggt ttc cat gtc cat gct
 L   K   P   G   L   H   G   F   H   V   H   A  48
ctt ggt gac acc act aat ggt tgc atg tcc acc ggt
 L   G   D   T   T   N   G   C   M   S   T   G  60
         ♦                       ●
ccg cat ttc aac cct gat ggt aaa acc cac ggt gca
 P   H   F   N   P   D   G   K   T   H   G   A  72
                                     ●
cca gag gat gct aat cgt cat gct gga gat cta gga
 P   E   D   A   N   R   H   A   G   D   L   G  84
aac ata act gtt ggg gat gat gga act gct tcc ttc
 N   I   T   V   G   D   D   G   T   A   S   F  96
aca atc act gac agc cag ata cct ctt gat gga cct
 T   I   T   D   S   Q   I   P   L   D   G   P 108
                                         ★
aac tct att gtt gga agg gct gtt gtt gtc cac gca
 N   S   I   V   G   R   A   V   V   V   H   A 120
gac cct gat gac ctc ggc aag gga ggc cat gaa ctc
 D   P   D   D   L   G   K   G   G   H   E   L 132
agc ttg gct act gga aat gca gga ggt cgt gtt gct
 S   L   A   T   G   N   A   G   G   R   V   A 144
tgt ggt att att ggt ctt cag ggc taa
 C   G   I   I   G   L   Q   G   *                152
gctgttgctattcg aggaagagag tgatgtaata aggagg
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(B) *RsFeSod*

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                                                tatca
aaggcatcctcaaacatagaactcaaaccacagatatcactggcgtg
tgctctcctcacttgccttgaaactagagcttcaatcccaaaaaaaa
atg gcc gct tca gct gct gta acc gca aac tac gtc
 M   A   A   S   A   A   V   T   A   N   Y   V  12
ctc aag cca cct cca tac cct ctg gat gct ttg gag
 L   K   P   P   P   Y   P   L   D   A   L   E  24
     ★
ccg cat atg agc aag caa act cta gag ttt cac tgg
 P   H   M   S   K   Q   T   L   E   F   H   W  36
gga aaa cac cac agg gct tac gtg gac aac ctc aag
 G   K   H   H   R   A   Y   V   D   N   L   K  48
aaa cag gtt ctt gga tcc gag ctt gaa ggc aag gcc
 K   Q   V   L   G   S   E   L   E   G   K   A  60
tta gag cat atc atc caa aac act tac aac aat ggc
 L   E   H   I   I   Q   N   T   Y   N   N   G  72
gac ctc ctc cct cct ttc aac aac gct gct cag gcg
 D   L   L   P   P   F   N   N   A   A   Q   A  84
         ★
tgg aac cac gag ttt ttc tgg gaa tca atg aaa cca
 W   N   H   E   F   F   W   E   S   M   K   P  96
ggt ggt gga gga aag cca tca gga gag ctt ctt gct
 G   G   G   G   K   P   S   G   E   L   L   A 108
ctg ctt gaa aga gat ttc act tct tat gag aag ttt
 L   L   E   R   D   F   T   S   Y   E   K   F 120
tat gat gag ttc aat gct gct gct gcc act cag ttt
 Y   D   E   F   N   A   A   A   A   T   Q   F 132
gga gct ggc tgg gcc tgg ctt gct tac gca gat aac
 G   A   G   W   A   W   L   A   Y   A   D   N 144
aaa ctc aaa gtt gtg aaa act cca aat gct gta aac
 K   L   K   V   V   K   T   P   N   A   V   N 156
ccc ctt gtg ctc ggc tct ttc cca ttg ctt acc att
 P   L   V   L   G   S   F   P   L   L   T   I 168
★               ★
gat gtc tgg gag cat gca tac tat ctc gac ttc cag
 D   V   W   E   H   A   Y   Y   L   D   F   Q 180
aac cgg aga ccc gat tac ata aag aca ttc atg aac
 N   R   R   P   D   Y   I   K   T   F   M   N 192
aat ctt gtg tct tgg gag gct gtt agt tcc aga ctt
 N   L   V   S   W   E   A   V   S   S   R   L 204
gag gct gcc aag gct gct tct gct taa
 E   A   A   K   A   A   S   A   *                212
gcagagtcatcagacacactcggaccaaaactctgacttcagttatg
tgtgttatgcattactgaagtttcttaattaaata
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Figure 1. Nucleotide and deduced amino acid sequences of *RsCu/ZnSod* (**A**) and *RsFeSod* (**B**). Amino acid residues (His-45, - 47, -62, - 70, -82, -119, and Asp-82) involved in Cu-(★), Cu/Zn-(◆), and Zn-(●) binding ligand (**A**), and those (His-26, -87, -173, and Asp-169) involved in Fe binding ligand (★) (**B**) are indicated. Nucleotide sequences used in PCR are underlined. GenBank accession numbers for *RsCu/ZnSod* and *RsFeSod* are **AF009735** and **AF061583**, respectively.

Now, to isolate the cDNA clones encoding Cu/ZnSOD and FeSOD from *R. sativus*, we PCR-amplified the cDNA strand synthesized from mRNA as template, using a pair of degenerate primers (Table 1). One band, corresponding to the expected sizes of 285 and 306 bp, was amplified and sequenced. The nucleotide sequences showed high homology with those of other species (data not shown). By screening our small radish cDNA library with these clones serving as probes, we finally identified the cytosolic *Cu/ZnSod* and *FeSod* clones and named them *RsCu/ZnSod* and *RsFeSod*, respectively (Fig. 1). *RsCu/ZnSod* encodes a protein of 153 amino-acid residues, with a molecular weight of 15.1 kDa and a pI of 5.44. Its deduced amino acid sequence shares the highest identity (94%) with that of the *Arabidopsis Cu/ZnSod*, as well as 82-85% identity with those of other plant species. The putative Cu-, Zn-, and Cu/Zn-binding ligands (Fig. 1A) are well conserved at His-45, 47, 62, 70, 82, 119, and Asp-82 of the deduced amino acid sequences (Kanematsu and Asada, 1994). The second clone, *RsFeSod*, is 823 bp long and encodes a protein of 213 amino acids, with a predicted molecular mass of 25.4 kDa and a pI of 8.77. Its deduced amino acid sequence shares the highest identity (93%) with that of *Arabidopsis FeSod*, and 60-73% identity with those of other species. The putative Fe-binding ligands (Fig. 1B) are well conserved at His-26, 87, 173, and Asp-169 of the deduced amino acid sequences (Kanematsu and Asada, 1994).

To investigate the copy number of SOD genes in the radish genome, cDNA fragments corresponding to part of the *RsCu/ZnSod* and *RsFeSod* coding regions served as probes for genomic DNA gel-blot analysis. Using the *RsCu/ZnSod* probe, we detected a few bands in each restriction enzyme digested genome, but only a single band was found in the *RsFeSod* probe reactions, suggesting that these SOD genes are encoded by a small (or single) gene family in the radish genome (data not shown). Similar results have been reported for *Cu/ZnSod* and *FeSod* in other plants (Shin et al., 2005).

SOD Isozyme Patterns at Different Developmental Stages and in Various Tissues

SOD activity in small radish was analyzed with an isozyme gel. When seedlings were grown under dark/light conditions for 8 d, several SOD isozymes were found at different growth stages. Early on, this activity did not vary between cytosolic Cu/ZnSOD and FeSOD, but treatment under lights prompted a slight change in MnSOD and also induced chloroplastic Cu/ZnSOD activity. In addition, a few upper bands were found in seedlings at 2 d post-germination (Fig. 2A). In our assay for tissue-specific SOD activity, several SOD isozymes were distributed within different tissues, indicating that multiple SOD genes were present, based on three metal cofactors, each encoding a distinct SOD isozyme (Fig. 2B). Generally, the cytosolic Cu/ZnSOD isozyme band appeared for all tested tissues, while the FeSOD and chloroplastic Cu/ZnSOD isozyme bands were associated with the leaves, the site of light-related functions. MnSOD activity was higher in the red hypocotyls and roots than in other organs. These amounts of SOD protein activity did not exactly match the level of transcript detected for

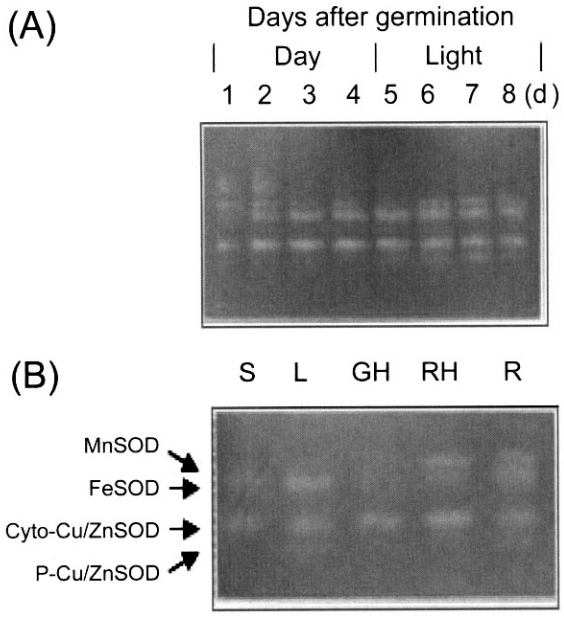

Figure 2. Activity of three RsSODs at early developmental stages (A) and in different organs (B). Samples (10 μg of crude soluble protein) isolated from seedlings were loaded onto 7% acrylamide gel. S, seedling; L, leaf; GH, green hypocotyl; RH, red hypocotyl; R, root.

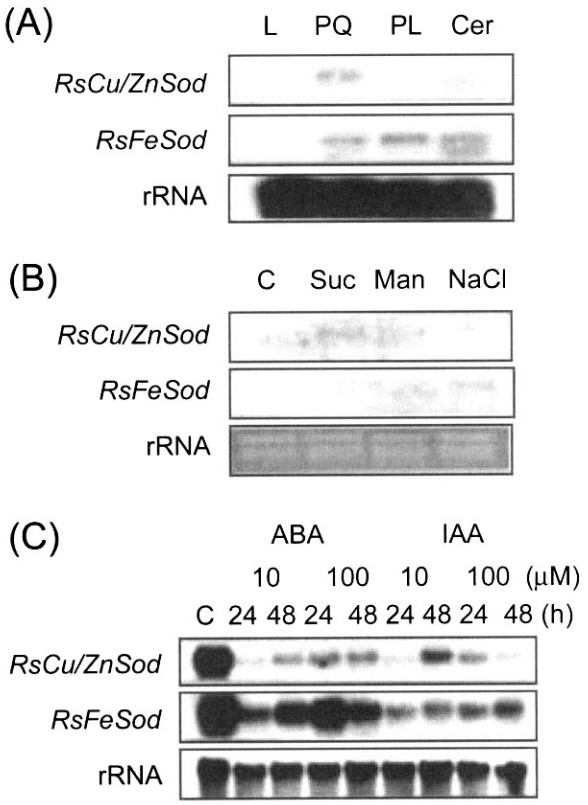

Figure 3. Northern analysis of *RsSod* gene expression in response to redox-cycling agents, osmoticums, and hormones. (A) Leaves of plants grown for two weeks under illumination were sprayed with 5×10^{-5} m paraquat (PQ), plumbagin (Plu), or cercosporin (Cer) for 24 h in light. (B) Etiolated seedlings were incubated for 24 h in MS medium supplemented with 10% sucrose, 10% mannitol (Mann), or 100 mM NaCl. (C) Etiolated seedlings were incubated on MS medium supplemented with 10 or 100 μM concentrations of ABA or IAA for 24 or 48 h under darkness. Total RNA (20 μg per lane) was separated by gel electrophoresis and blotted onto nylon membranes.

each SOD gene, which had been determined previously (Kwon and An, 2003). Although all three SOD genes are expressed in green hypocotyls and leaves, through to varying degrees, only *RsMnSod* is highly expressed in the roots (Kwon and An, 2003). These observations are similar in other plant species, including rice. Although the rice SOD genes are differentially expressed in the etiolated seedlings, leaves, stems, and roots, their levels of transcripts are quite different from that measured for the rice SOD isozyme (Kaminaka et al., 1997, 1999). Therefore, we propose that regulation of the SOD gene is controlled by different transcriptional or translational mechanisms.

Effect of Xenobiotics, Osmoticums, and Hormones on SOD Transcripts

Xenobiotics accept electrons from transport chains in the chloroplasts or mitochondria, thereby increasing cellular ROS. Cercosporin, which becomes a photoactivated polyketide toxin when activated by light, is converted to an electronically excited triplet state that can react with oxygen to produce ROS species (Scandalios, 1997). To investigate the regulation of SOD genes in response to xenobiotics, we tested different compounds known to increase cellular ROS concentrations to determine their effect on *SOD* transcript levels. After being sprayed with paraquat (PQ), plumbagin (Plu), or cercosporin (Cer), the SOD transcripts changed in all treated leaves (Fig. 3A). However, although *RsFeSod* was induced after all three treatments, *RsCu/ZnSod* was strongly induced only by PQ and was just weakly expressed in response to Plu and Cer. Previously, we had reported that *RsMnSod* is induced by PQ and Cer (Kwon and An, 2003). This induction pattern has also been found in maize and tobacco (Tsang et al., 1991; Williamson and Scandalios, 1992). Therefore, we might surmise that, although the combined action of xenobiotics and light impose an oxidative stress mainly on the chloroplasts, both mitochondrial *RsMnSod* and cytosolic *RsCu/ZnSod* transcripts also are induced to protect cellular compartments from oxidative damage, in addition to this role played by *RsFeSod*.

To understand the effect of osmoticums on the induction of SOD transcripts, we analyzed the expression of *RsCu/ZnSod* and *RsFeSod* following treatment. Although the level of *RsMnSod* transcript was increased by all applications (Kwon and An, 2003), neither *RsCu/ZnSod* nor *RsFeSod* were rarely affected (Fig. 3B). To gain insight into the regulation of SOD genes by phytohormones, seedlings were also exposed to ABA or IAA (Fig. 3C). For ABA, *RsFeSod* was induced to a greater degree over time than was *RsCu/ZnSod*, showing a stronger increase in response at the low concentration (10 μM), but being slightly decreased at the high concentration (100 μM). In contrast, *RsCu/ZnSod* expression was induced slightly over time by the low concentration (10 μM), but not changed at the higher application (100 μM). The opposite trend was detected in response to IAA treatment, with *RsFeSod* expression not being

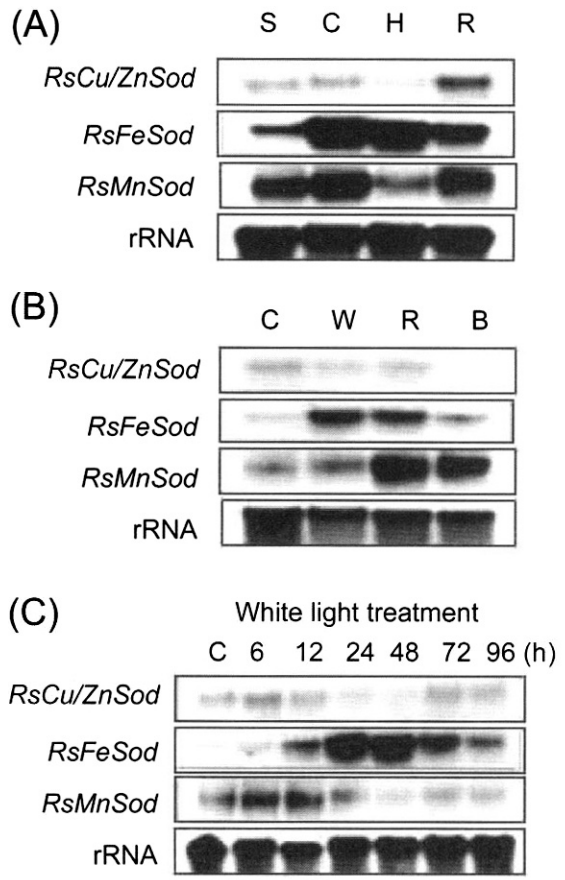

Figure 4. Northern analysis of *RsSod* genes in response to various lighting conditions. (**A**) Expression of three genes in different organs of small radish treated with white light for 48 h. S (seedling), C (cotyledon), H (hypocotyls), and R (root). (**B**) Expression of three genes in hypocotyls of seedlings treated with white light (W), red light (R), or blue light (B) for 3 d (C; Control). (**C**) Expression of three genes in hypocotyls of seedlings treated with white light for times indicated.

changed over time, regardless of the concentration tested, in contrast to *RsCu/ZnSod* expression, which rose over time at the low concentration but declined at the high concentration. Previously, we had reported that *RsMnSod* expression also increased in response to ABA and IAA (Kwon and An, 2003). Similar trends have been described for *Cu/ZnSod* and *FeSod* expressions in tomato, barley, soybean, and tobacco (Crowell and Amasino, 1991a; Perl-Treves and Galun, 1991; Casano et al., 1994; Zhu and Scandalios, 1994; Kurepa et al., 1997). Thus, we are certain that the SOD genes are induced by certain redox-cycling agents (xenobiotics) that are known to cause oxidative stress, and that they function as a cross-link interaction between the hormone and the defense response to that stress.

Differential Expression of SOD Transcripts in Response to Various Light Sources

The level of endogenous ROS production is positively correlated with the incidence of light during plant growth. Therefore, we first used northern blot analysis to characterize the tissue-specific expression of radish SOD genes under various illumination conditions (Fig. 4A). When hypocotyls were exposed to white light for 48 h, the three SOD transcripts were detected in all the tissues examined, with *RsCu/ZnSod* being most strongly induced in the roots, only a little in the whole seedling and cotyledons, and just rarely in the hypocotyls. *RsFeSod* was highly expressed in all tested tissues compared with *RsMnSod*, which was detected at nearly equal levels in the whole seedling, cotyledons, and roots, but only weakly in the hypocotyls. In a second set of experiments, we assessed how SOD gene expression in the hypocotyls was affected by white, red, or blue light (Fig. 4B). The transcript level for *RsCu/ZnSod* did not differ much from that of the control, suggesting that cytosolic *RsCu/ZnSod* may not be involved in protecting against oxidative stress that originates from various light sources. In contrast, *RsFeSod*, which may be located in the chloroplast, was strongly influenced by white and red light, such that we might conclude that this gene plays a role in detoxifying photosynthesis-mediated oxidative stresses. Finally, *RsMnSod* was strongly induced by red and especially blue light, implying that this gene is related to the detoxifying function of oxidative stresses under those conditions.

We used northern blotting to analyze the expression pattern of SOD genes when hypocotyls were treated over time with white light (Fig. 4C). At first, *RsCu/ZnSod* and *RsMnSod* showed similar expression patterns, being highly induced early on (until 12 h) before decreasing by the mid point of the test period (from 24 to 48 h), and then being slightly induced again after 72 h. This pattern was reversed for *RsFeSod*, which was most strongly induced from 12 to 72 h. Based on these data, we can confirm that the expression of SOD genes is differentially regulated to meet certain functions in a time-dependent manner, and that their activity is related to their functional localization in the cell (cytosol/mitochondria or chloroplast). Transcript levels for genes for chloroplastic Cu/ZnSOD and FeSOD that are higher during the illuminated period than under darkness have been reported in *Arabidopsis* (Kliebenstein et al., 1998), tobacco (Kurepa et al., 1997), rice (Kaminaka et al., 1999), and liverwort (Sakaguchi et al., 2004). However, *Arabidopsis* microarray analysis did not indicate any upregulated or downregulated expression of the SOD genes in the profiles of seedlings grown under different light sources, results which could have confirmed the role of photoreceptors during photomorphogenesis (Ma et al., 2001). Thus, our findings suggest that radish SOD genes are complementarily expressed with other cellularly localized SOD genes in various organs, and that expression is differentially induced by special light signals.

Accumulation of SOD Transcripts in Response to UV and SA Treatments

UV-B causes oxidative stress in plants, leading to ROS production through the activation of UV-B chromophores (Willekens et al., 1994; Landry et al., 1995). A possibly different mechanism for the oxidative stress response prompted us to examine the expression pattern of SOD genes during UV exposure. *RsCu/ZnSod* transcript was increased slightly during the test period, while that of *RsFeSod* did not differ significantly between the treated plants and the untreated controls (Fig. 5A). In our previous work with catalase and other antioxidant enzymes, three separate transcripts were

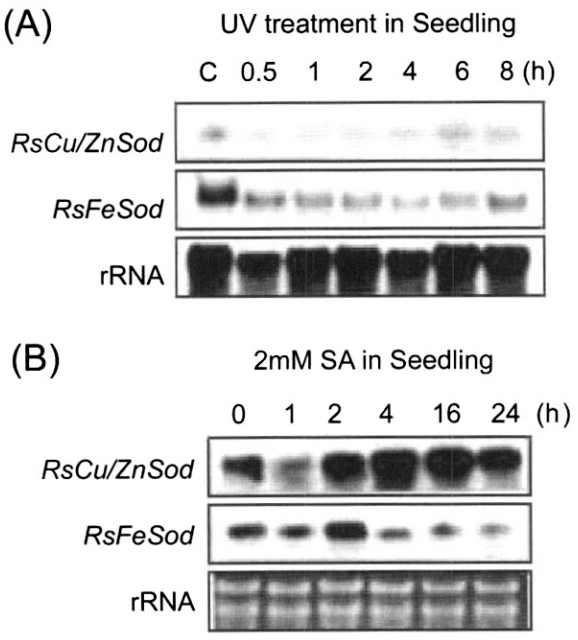

Figure 5. Northern analysis of *RsSod* gene expression in small radish seedlings in response to treatment with UV light (**A**) or SA (**B**).

differentially induced by UV treatment (unpublished data). From experiments using *Arabidopsis* seedlings (Kliebenstein et al., 1998), the same results were obtained, with induction being slight for *CDS1*, a *Cu/ZnSod* gene, but nonexistent for *FeSod* and *MnSod*. Thus, we might speculate that UV treatment generates plant oxidative stress by very different mechanisms. This general responsiveness also might suggest that cytosolic SOD is a general stress-response enzyme.

Salicylic acid (SA) is a critical hormone for signaling innate immunity in plants. Several putative effector proteins have been identified in tobacco, including catalase and ascorbate peroxidase. To characterize the regulation of SOD transcripts in response to SA, we treated radish seedlings with 2 mM SA, and found that *RsCu/ZnSod*, but not *RsFeSod*, was induced (Fig. 5B). Similar expression patterns and the conclusion that SOD protein accumulation is regulated in this manner have been reported in *Arabidopsis* (Kliebenstein et al., 1999). Likewise, the expression of chloroplastic *Cu/ZnSod* in the needles of maritime pine (*Pinus pinaster*) is up-regulated by salicylic acid (Azevedo et al., 2004). Thus, we suggest that SA-mediated signals induce the expression of both cytosolic and chloroplastic *Cu/ZnSod*, and that the subsequent accumulation of Cu/ZnSOD dismutates any O_2^- before the ROS signals a transition to cell damage.

In conclusion, to ascertain the structure and expression pattern of SOD genes in small radish, we have now isolated SOD cDNA clones that are differentially expressed in a tissue-specific manner and, over time, in response to various abiotic treatments. These *RsSod* genes can be used as molecular probes to study oxidative stresses that occur specifically in the plant cytoplasm, mitochondria, and chloroplast because of environmental adversity. Based on our results, we can report that the *RsSod* genes are associated with detoxifying functions, which remove the ROS that occurs during metabolic activity in different cellular compartments. Further experiments might focus on transgenic *Arabidopsis* plants that over-express these three SOD genes in response to various stresses. By studying their expression patterns and associated enzyme activities, researchers should be able to clarify the overall importance of SOD genes to various oxidative stress phenomena in small radish.

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