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Expression of a Cu-Zn Superoxide Dismutase Gene in Response to Stresses and Phytohormones in *Rehmannia Glutinosa*

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ABSTRACT : Superoxide dismutases (SOD) are metalloenzymes that convert O_2^- to H_2O_2. *Rehmannia glutinosa* is highly tolerant to paraquat-induced oxidative stress. The primary objective of this study was to characterize regulation of *SOD* gene expression in *R. glutinosa* in response to oxidative stresses and hormones. A full-length putative SOD clone (*RgCu-ZnSOD1*) was isolated from the leaf cDNA library of *R. glutinosa* using an expressed sequence tag clone as a probe. *RgCu-ZnSOD1* cDNA is 777 bp in length and contains an open reading frame for a polypeptide consisted of 152 amino acid residues. The deduced amino acid sequence of the clone shows highest sequence similarity to the cytosolic Cu-ZnSODs. The two to three major bands with several minor ones on the Southern blots indicate that *RgCu-ZnSOD1* is a member of a small multi-gene family. *RgCu-ZnSOD1* mRNA was constitutively expressed in the leaf, flower and root. The expression of *RgCu-ZnSOD1* mRNA was increased about 20% by wounding and paraquat, but decreased over 50% by ethylene and GA_3. This result indicates that the *RgCu-ZnSOD1* expression is regulated differentially by different stresses and phytohormones at the transcription level. The *RgCu-ZnSOD1* sequence and information on its regulation will be useful in investigating the role of SOD in the paraquat tolerance of *R. glutinosa*.

***Key words* :** Cu-Zn superoxide dismutase (Cu-ZnSOD), oxidative stress, paraquat, *Rehmannia glutinosa*

INTRODUCTION

Paraquat (1,1'-dimethyl-4,4'-bipyridilium) is a post-emergence non-selective contact herbicide. Divalent paraquat cation produces free radicals which can combine with molecular oxygen to form reactive oxygen species (ROS; Dodge, 1994). These ROS can inhibit photosynthetic activity and cause peroxidation of the fatty acid side chains of membrane lipids, eventually leading to plant death (Scandalios, 1993).

Evolution of resistance to paraquat in susceptible plant species has been reported at least in 23 plant species, notably including *Conyza* species (http://www.weedscience.org/). Well documented paraquat resistance mechanisms include the detoxification of the ROS by the elevated antioxidant system (Shaaltiel, 1988) and rapid sequestration of the herbicide either by adsorption to cellular components or by transport in vacuole (Fuerst & Vaughn, 1990). Plants are equipped with a number of enzymatic and non-enzymatic ROS detoxifying agents to cope with oxidative stresses generated by biotic and abiotic stresses. Increased antioxidant enzyme levels are significantly associated with paraquat resistance in plant species, like *Lycopersicum* species (Thomas & Pratt, 1982), *Lolium perennae* (Harper & Harvey, 1978), *Conyza banariensis* (Shaalteil & Gressel, 1986), and *Pisum sativum* (Donahue *et al.*, 1997). In these species, resistant biotypes were emerged from susceptible populations.

Recently, paraquat tolerance was observed in *Rehmannia glutinosa*, a medicinal herb cultivated in many Asian countries (Kim & Chun, 1992; Chun *et al.*, 1997). A unique feature of the tolerance in *R. glutinosa*, however, is that tolerance is universal in the population of the species with no susceptible plants. Currently, the elevated antioxidant system (Choi *et al.*, 2004) and paraquat metabolism (Chun *et al.*, 1997) are proposed as a part of the tolerance mechanism. In our previous comparative enzymatic studies, we consistently observed considerably higher intrinsic levels and increased relative activities of superoxide dismutases (SOD; EC 1.15.1.1) under oxidative stress conditions in *R. glutinosa* (Choi *et al.*, 2004; Moon *et al.*, 2004). Regarding paraquat tolerance, special attention has been given to SOD, which are key elements of the antioxidant system as they detoxify superoxide radical to O_2 and H_2O in the first cellular defense against ROS (Bowler *et al.*, 1992). Since no molecular information is available on SOD of *R. glutinosa*, we attempted to isolate SOD genes and characterize

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their expression patterns in response to various stresses and stress-related phytohormones.

MATERIALS AND METHODS

Plant materials, bacterial strains, enzymes and chemicals

R. glutinosa (accession Kemsan) plants were grown in a greenhouse at Chonbuk National University, Korea. Three-month-old leaves collected by cutting the upper part of petioles with a razor blade were used for the treatment of stresses and hormone. The leaves with petioles were immersed in the following sterile solutions for 24 h at 20℃ under light: H_2O, 0.1 mM paraquat, 5 mM H_2O_2, 5 mM ethephon, 5 mM salicylic acid, 5 mM GA_3, or 25 ㎎/㎖ yeast extract (Chung *et al.*, 2003). For wounding treatments, the leaves were punched with fine pins and floated on the sterile water. For UV treatment, plants were irradiated under UV lamps at 1.35 $\mu E/m^2/s$ for 2 h and returned to the dark glasshouse. The irradiated leaves were collected 12 h after irradiation (Chung *et al.*, 2003). All tissue samples were frozen in liquid nitrogen and stored at −70℃ until used.

Escherichia coli strain XL1 Blue (Stratagene, USA), JM109 (Promega, USA) and Qiagen EZ (Qiagen, USA) were used for cloning according to the standard techniques (Sambrook & Russell, 2001). DNA modifying enzymes were purchased from TaKaRa (Japan) and Promega (USA), reagents for plant DNA and RNA extractions from MRC (USA), kits for plasmid DNA purifications from Bioneer (Korea) and other chemicals from Sigma (USA) unless otherwise indicated.

Cloning and DNA sequence analysis

A cDNA library of *R. glutinosa* leaf was constructed using Lamda ZAP cDNA library construction Kit (Stratagene, USA) according to the manufacturer's instruction. Expressed sequence tags (ESTs) were generated by the random sequencing of the insert DNAs of the plasmids excised from the lamda cloning vector. An EST showing sequence homology to the known plant SODs was used as a probe to screen the library. SOD-positive clones were isolated by screening 5.6×10^9 *pfu* of the cDNA library and the insert DNA of a clone was subcloned into pBluscript SK(+) for nucleotide sequence determination. The nucleotide sequences of both strands of the insert DNA were determined by the dideoxy chain termination method. Homology searches of databases were performed using the BLAST programs (Altschul *et al.*, 1997) against DNA and protein sequences. Nucleotide and deduced amino acid sequence analyses were performed using DNASIS (Hitachi, USA) and the programs and databases offered by the National Center for Biotechnology Information (NIH, USA) and European Bioinformatics Institute (EBI, UK). Multiple sequence analysis was performed using the program AliBee (GeneBee, SU).

Southern and northern blot analysis

Genomic DNA was prepared from the leaves using the plant DNA extraction kit (DNA zol ES, MRC, USA) according to manufacturer's instruction. DNA concentration was estimated by subjecting samples to 0.8% agarose gel electrophoresis and staining with ethidium bromide. Staining intensities of the total DNA were compared visually with a DNA molecular weight marker. Southern blot analysis was carried out with genomic DNA as described (Sambrook & Russell, 2001). Genomic DNA digested to completion with the restriction enzymes, *Eco*R V and *Kpn* I and *Sca* I was separated by agarose gel electrophoresis and transferred onto a nylon membrane (Hybond-N^+, Amersham, UK). Labeling and detection were conducted with AlkPhos Direct system (Amersham Pharmacia biotech, UK). Hybridization, washing, signal generation and detection were performed with a chemiluminescent system (CDP-*Star*, Amersham Pharmacia biotech, UK) (Cho *et al.*, 2005).

Total RNA was extracted using the TRI reagent procedure (MRC, USA). For northern blot analysis, total RNA (20 ㎍) was denatured, separated on a 1% formaldhyde-gel, and transferred onto a nylon membrane (Hybond-N^+, Amersham, UK). The membrane was prehybridized for 1 h at 60℃ in the AlkPhos Direct hybridization buffer (500 mM NaCl, 0.4% blocking reagent) and incubated for 16 h at 60℃ after adding the labeled probe in the prehybrization solution. After hybridization the membrane was washed and mRNA on the membrane was detected as in Southern blot.

RESULTS AND DISCUSSION

Sequence analysis of *RgCu-ZnSOD1*

A putative full-length cDNA clone encoding SOD polypeptide (*RgCu-ZnSOD1*) was isolated using an EST clone showing high sequence similarity to the known plant Cu-ZnSODs. The nucleotide and deduced amino acid sequences are shown in Fig. 1. *RgCu-ZnSOD1* is consisted of 777 bp nucleotides containing an ORF and a 5' and a 3' untranslated regions.

The deduced amino acid sequences share the conserved structural features among plant CuZn-SODs such as the residues required for binding copper (H-45, -47, -62, and -119) and zinc (H-62, -70 and -79, and D-82). Two cysteine residues at the positions 56 and 145 are predicted to form a single disulfide bond. The two cysteine residues are also conserved in plant Cu-ZnSODs (Fridovich, 1986).

A multiple sequence alignment reveals that the deduced amino

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1   ATG GGT ACT AAT ACG GGC TCA CGC GGT GGC GGC CGC TCT AGA ACT AGT GGA TCC CCC GGG CTG CAG GAA TTC   72
73  GGC ACG AGG AAC AGG GGT GCT CTG AGA TCA CAA TTA ACC ATG GTG AAG GCT GTC GCA GTG CTC AAC AGT AGT  144
                                                     M   V   K   A   V   A   V   L   N   S   S    11
145 GAG GGT GTT AGT GGC ACC ATC TAC TTC ACC CAG GAA GGA GAT GGT CCG ACA ACT GTT ACT GGA AAC CTT TCT  216
12   E   G   V   S   G   T   I   Y   F   T   Q   E   G   D   G   P   T   T   V   T   G   N   L   S    35
217 GGC CTT AAG CCT GGA CAA CAT GGC TTC CAT GTG CAT GCC CTT GGT GAC ACC ACC AAT GGT TGT ATG TCT ACT  288
36   G   L   K   P   G   Q   H   G   F   H   V   H   A   L   G   D   T   T   N   G   C   M   S   T    59
289 GGA CCT CAC TTC AAT CCT GCC GGC AAG GAA CAT GGT GCT CCT GAT GAT GAG GTT CGC CAT GCT GGT GAC CTT  360
60   G   P   H   F   N   P   A   G   K   E   H   G   A   P   D   D   E   V   R   H   A   G   D   L    83
361 GGG AAT GTC ACA GTT GGA GAA GAT GGC AAA GCT GAT TTC ACC ATT GTT GAC AAG CAG ATA CCA CTT ACA GGA  432
84   G   N   V   T   V   G   E   D   G   K   A   D   F   T   I   V   D   K   Q   I   P   L   T   G   107
433 CCA CAT TCT ATA ATT GGA AGA GCT GTA GTT GTC CAT GCT GAT CCG GAT GAT CTT GGA AAG GGT GGA CAT GAA  504
108  P   H   S   I   I   G   R   A   V   V   V   H   A   D   P   D   D   L   G   K   G   G   H   E   131
505 CTA AGC AAA ACC ACT GGA AAT GCT GGA GGA AGA GTT GCT TGT GGA ATC ATT GGC CTT CAG GGC TGA GTA AAC  576
132  L   S   K   T   T   G   N   A   G   G   R   V   A   C   G   I   I   G   L   Q   G               152
577 CCG GTG GCG TGC TCT ACT TTC GTT GTT GCT TAT ATC TTT TCT CCG GGA GAA GGA TCC TAC ATC ACT TGC TAC  648
649 CTA TTG GGT TCT TAA ATA AAG GCT TTG TTA GAA TGA ACG TTT TTC ATA TGT AAT TCT ACA TAT AAG TCG TAT  720
721 GGT TTA CTG AGT ACT ATA CCT ATT GAA AGT CTG ATA CCG TTA TTC TAA AAA AAA AAA                      777
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Fig. 1. The nucleotide and deduced amino acid sequence of *RgCu-ZnSOD1* cDNA clone. The residues binding copper (H-45, -47, -62, and -119) are double-underlined and those binding zinc (H-62, -70, and -79, and D-82) underlined. The cysteine residues forming a single disulfide bond are boxed.

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SlCu-ZnSOD    -----MAAHTILASAPSHTTFSLISPFSSTPTNALSSSLQSSSFNGLSFKLSPTTQS-LSLSTSAASKPLTIVAATKKAVAVLKGTSNVE 83
BuCu-ZnSOD    -------------------------------------------------------------MPMLAVAKSRTLTITAATKKAVAVLKGTSNVE 32
PsCu-ZnSOD2   -----MASQTLVSPS----------PLSSHS-------LLRTSFSGVSVKLAPQFST----LATSNFKPLTVVAAAKKAVSVLKGTSAVE 64
SlCu-ZnSOD2   -----MAAHSIFTTTS--TTNSFLYPISSSSS----SPNINSSFLGVSLNVNAKFGQSLTLYAVTTPKPLTVFAATKKAVAVLKGNSNVE 79
ZmCu-ZnSOD    ----------------------------------------------------------------------MV--KAVAVLAGTD-VK 14
OsCu-ZnSOD    ----------------------------------------------------------------------MV--KAVAVLASSEGVK 15
RgCu-ZnSOD1   ----------------------------------------------------------------------MV--KAVAVLNSSEGVS 15
SlCu-ZnSOD1   ----------------------------------------------------------------------MV--KAVAVLNSSEGVS 15
IbCu-ZnSOD    ----------------------------------------------------------------------MV--KAVAVLSSSEGVS 15
SoCu-ZnSOC    ----------------------------------------------------------------------MG--KAVVVLSSSEGVS 15
AtCu-ZnSOD    ----------------------------------------------------------------------MA--KGVAVLNSSEGVT 15
CpCu-ZnSOD    ----------------------------------------------------------------------MV--KAVAVLSSSEGVS 15
PsCu-ZnSOD1   ----------------------------------------------------------------------MV--KAVAVLSNSNEVS 15
PsCuZnSOD     ----------------------------------------------------------------------MAGLKAVAVPSGSENVK 17
LsFeSOD       MAATASANSLTSAFLPPQGFNGSSKSLQWRTQKKQFGRKAGSATITAKFDLIPPPYPMDALEPHMSSRTFEFHWGKYHRAYVDNLNKQID 90
BuFeSOD       MAAVP---------LP------------------------LTTVDLRPPPYALDALEPHMSKETLEYHWGKHHRAYVDNLKKQIE 52

SlCu-ZnSOD    GVVTLTQEDDGPTTVNVR----ISGLAPGKHGFHLHEFGDTTN---GCMSTGPHFNPDKKTHGAPEDEVRHAGDLGNIVANT-------- 159
BuCu-ZnSOD    GVVTLLQEDDGPTKVNVK----ITGLAPGKHGFHLHEFGDTTN---GCMSTGPHFNPEGKTHGAPEDSNRHAGDLGNVIAGD-------- 107
PsCu-ZnSOD2   GVVTLTQDDEGPTTVNVR----ITGLTPGLHGFHLHEYGDTTN---GCISTGPHFNPNKLTHGAPEDEIRHAGDLGNIVANA-------- 139
SlCu-ZnSOD2   GVVTLSQDDDGPTTVNVR----ITGLAPGLHGFHLHEYGDTTN---GCMSTGAHFNPNKLTHGAPGDEIRHAGDLGNIVANA-------- 154
ZmCu-ZnSOD    GTIFFSQEGDGPTTVTGS----ISGLKPGLHGFHVHALGDTTN---GCMSTGPHFNPVGKEHGAPEDEDRHAGDLGNVTAGE-------- 89
OsCu-ZnSOD    GTIFFSQEGDGPTSVTGS----VSGLKPGLHGFHVHALGDTTN---GCMSTGPHFNPTGKEHGAPQDENRHAGDLGNITAGA-------- 90
RgCu-ZnSOD1   GTIYFTQEGDGPTTVTGN----LSGLKPGQHGFHVHALGDTTN---GCMSTGPHFNPAGKEHGAPDDEVRHAGDLGNVTVGE-------- 90
SlCu-ZnSOD1   GTYLFTQVGVAPTTVNGN----ISGLKPGLHGFHVHALGDTTN---GCMSTGPHYNPAGKEHGAPEDEVRHAGDLGNITVGE-------- 90
IbCu-ZnSOD    GTIFFSQEGDGPTTVTGN----VSGLKPGLHGFHVHALGDTTN---GCMSTGPHFNPAGKEHGAPGDDNRHAGDLGNITVGE-------- 90
SoCu-ZnSOC    GTVYFAQEGDGPTTVTGN----VSGLKPGLHGFHVHALGDTTN---GCMSTGPHYNPNGKEHGAPEDDVRHAGDLGNITVGD-------- 90
AtCu-ZnSOD    GTIFFTQEGDGVTTVSGT----VSGLKPGLHGFHVHALGDTTN---GCMSTGPHFNPDGKTHGAPEDANRHAGDLGNITVGD-------- 90
CpCu-ZnSOD    GTIFFTQAADGPTTVTGE----ISGLKPGHHGFHVHALGDTTN---GCMSTGPHFNPAGKEHGAPEDDIRHAGDLGNVNVGD-------- 90
PsCu-ZnSOD1   GTINFSQEGNGPTTVTGT----LAGLKPGLHGFHIHALGDTTN---GCISTGPHFNPNGKEHGAPEDETRHAGDLGNINVGD-------- 90
PsCuZnSOD     GVLHFTQEGNEATKVFGR----ITGLKPGLHGFHVHSMGDTTN---GCLSTGPHYNPWKKDHGAPEDENRHAGDLGNIIAGE-------- 92
LsFeSOD       GTELDGKTLEDIILVTYNNGAPLPAFNNAAQAWNHQFFWESMKPNGGGEPSGELLELINRDFGSYDTFVKEFKAAAATQFGSGWAWLAYK 180
BuFeSOD       GTELASQTLEDIVRATYNNGEPTAPFNNAAQAWNHEFFWLSMSPHGGKQPDGELMSLLKRDFGSYDNFVKEFKQAGATQFGSGWAWLTVA 142

SlCu-ZnSOD    --DGVAEATIVDNQIP--LTGPNSVVGRALVVHELEDDLGKGGHELSPTTGNAGGRLACGVVGLTPV---- 222
BuCu-ZnSOD    --DGVVEVELQDSQIP--LSGPHSVVGRAFVIHEAEDDLGKGGHELSSTTGNAGGRLACGVVGLTPL---- 170
PsCu-ZnSOD2   --EGVAEATIVDNQIP--LTGPNSVVGRALVVHELQDDLGKGGHELSLSTGNAGGRLACGVVGLTPV---- 202
SlCu-ZnSOD2   --DGVAEVTLVDNQIP--LTGPNSVVGRALVVHELEDDLGKGGHELSLTTGNAGGRLACGVVGLTPI---- 217
ZmCu-ZnSOD    --DGVVNVNITDSQIP--LAGPHSIIGRAVVVHADPDDLGKGGHELSKSTGNAGGRVACGIIGLQG----- 151
OsCu-ZnSOD    --DGVANVNVSDSQIP--LTGAHSIIGRAVVVHADPDDLGKGGHELSKTTGNAGGRVACGIIGLQG----- 152
RgCu-ZnSOD1   --DGKADFTIVDKQIP--LTGPHSIIGRAVVVHADPDDLGKGGHELSKTTGNAGGRVACGIIGLQG----- 152
SlCu-ZnSOD1   --DGTASFTITDKQIP--LTGPQSIIGRAVVVHADPDDLGKGGHELSKSTGNAGGRIACGIIGLQG----- 152
IbCu-ZnSOD    --DGTASFTITDKQIP--LTGANSVIGRAVVVHGDPDDLGKGGHELSKSTGNAGGRVACGIIGLQG----- 152
SoCu-ZnSOC    --DGTATFTIIDSQIP--LSGPNSIVGRAVVVHAEPDDLGRGGHELSKTTGNAGGRVACGIIGLQG----- 152
AtCu-ZnSOD    --DGTATFTITDCQIP--LTGPNSIVGRAVVVHADPDDLGKGGHELSLATGNAGGRVACGIIGLQG----- 152
CpCu-ZnSOD    --DGKVSFSIIDSQIP--LTGPNSIVGRAVVVHADPDDLGKGGHELSKTTGNAGGRVACGVIGLQG----- 152
PsCu-ZnSOD1   --DGTVSFTITDNHIP--LTGTNSIIGRAVVVHADPDDLGKGGHELSKTTGNAGGRVACGIIGLQG----- 152
PsCuZnSOD     --DGVAELSIQDWKIP--LKGPHSIVGRAVVVHADRDDLGRGGHELSKTTGNAGGRVACGVIGLQSAV--- 156
LsFeSOD       PEDKKLALVKTPNAENPLVLGYTPLLTIDVWEHAYYLDFQNRRPDYISIFMEKLVSWEAVSIRLKAASA-- 249
BuFeSOD       --DGKLMVEKSPNAINPLVFGHVPILVADVWEHAYYLDYQNRRPDYLTTFMNELVSWDAVAKRLQLALQES 211
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Fig. 2. Alignment of amino acids sequences of plant superoxide dismutases. Amino acid sequences were deduced from *SOD* cDNA clones from *Arabidopsis thaliana* (AtCu-ZnSOD, X60935), *Barbula unguiculata* (BuCu-ZnSOD, AB066500; BuFeSOD, AB066499), *Carica papaya* (CpCu-ZnSOD, Y13610), *Ipomea batatas* (IbCu-ZnSOD, X73139), *Lycopersicon esculentum* (LeFeSOD, AY262025), *Oryza sativa* (OsCu-ZnSOD, D01000), *Pinus sylvestris* (PsCu-ZnSOD, AJ307586), *Pisum sativum* (PsCu-ZnSOD1, M63003; PsCu-ZnSOD2, J04087), *Solanum lycopersicum* (SlCu-ZnSOD1, X14040; SlCu-ZnSOD2, X14041), *Spinacia oleracea* (SoCu-ZnSOD, D10244), *Spinacia oleracea* (SoCu-ZnSOD, X53872), and *Zea mays* (ZmCu-ZnSOD, M15175). GenBank accession number for each clone is indicated.

acid sequence encoded by *RgCu-ZnSOD1* shows higher homology to the known cytosolic Cu-ZnSODs (cytCu-ZnSOD) than chloroplastic Cu-ZnSODs (chlCu-ZnSOD) or FeSODs (Fig. 2). Most of the copper and zinc binding residues and disulfide binding sequences are conserved among Cu-ZnSODs but not among FeSODs (Fig. 2). RgCu-ZnSOD1 also shows close evolutionary relationship with cytCu-ZnSODs than chlCu-ZnSODs (Fig. 3). Thus, the sequence analysis results strongly suggest that RgCu-ZnSOD1 belongs to cytCu-ZnSOD. RgCu-ZnSOD1 shows a closest evolutionary relationship with tomato cytCu-ZnSODs (Fig. 3). The chlCu-ZnSODs are estimated to be evolved from cytCu-ZnSODs by gene duplication and acquisition of a transit peptide sequence (Tanaka *et al.*, 1996; Landis & Tower, 2005).

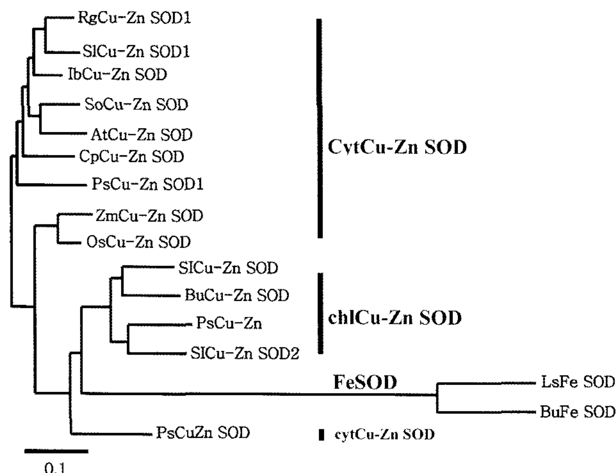

Fig. 3. Phylogenetic relationship of RgCu-ZnSOD1 with other plant SODs. Amino acid sequences were aligned using the CLASTAL W program of European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/) and the phylogenetic tree was constructed based on the distance calculated by the CLASTAL W multiple alignment. Abbreviations for the sequences are the same as in Fig. 2. cytCu-ZnSOD, cytosolic Cu-ZnSOD; chlCu-ZnSOD, chloroplastic Cu-ZnSOD.

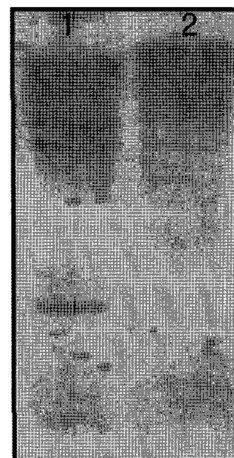

Fig. 4. Southern blot analysis of *RgCu-ZnSOD1* with genomic DNA digested with *Eco*R V (1) and *Kpn* I+*Sca* I (2), respectively. Ten micrograms of DNA were separated on a 0.8% (w/v) agarose gel and transferred to a nylon membrane, then hybridized with an alkaline phosphatase labeled *RgCu-ZnSOD1* insert DNA. The blot was washed to a final stringency of 0.1% SDS and 0.2% blocking reagent at 60℃ for 20 min.

Genetic constitution of *RgCu-ZnSOD1*

The genomic constitution of *RgCu-ZnSOD1* gene was estimated by probing Southern blots of genomic DNA digested with restriction enzymes that do not cut the insert DNA of the clone. Two to three major bands and several more minor ones on Southern blots indicate that *RgCu-ZnSOD1* belongs to a member of a small multigene family (Fig. 4). In many plant species, several *cytCu-ZnSODs* isoforms are often found but only with a single or no *chlCu-ZnSOD* (Baum *et al.*, 1983; Kanematsu & Asada, 1989a,b, 1990; Sen Gupta *et al.*, 1993; Streller *et al.*, 1994).

Regulation of *RgCu-ZnSOD1* gene expression

The expression of *RgCu-ZnSOD1* was investigated in the different tissues of *R. glutinosa* plant. *RgCu-ZnSOD1* mRNA was detected in the leaf, flower and root of healthy plants. The expression levels were about similar in all tissues (Fig. 5). Contrary to *RgCu-ZnSOD1* expression in all tissues, tissue-specific and inducible expression of *SOD* genes has also been reported in many plant species. *Cu-ZnSOD* genes of spinach (Ogawa *et al.*, 1997), tobacco (Herouart *et al.*, 1994), and pine (Karpinska *et al.*, 2001) are expressed differentially in various tissues. In tomato, chloroplast *Cu-ZnSOD* transcript is rare or absent from roots, stems and ripening fruits. In contrast, chloroplast *Cu-ZnSOD* transcript is very abundant in these organs (Perl-Treves & Galun, 1991).

To better understand the regulation of *RgCu-ZnSOD1* gene

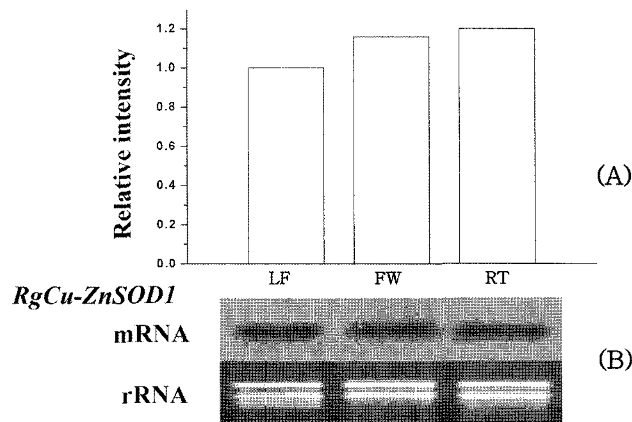

Fig. 5. Expression of *RgCu-ZnSOD1* mRNA in leaf (LF), flower (FW), and root (RT). (A) Relative intensity histograms. (B) Northern blot analysis. Twenty micrograms of total RNA were resolved on a 1.0% (w/v) agarose/formaldehyde gel and transferred to an nylon membrane, then hybridized with an alkaline phosphatase labeled *RgCu-ZnSOD1* insert DNA. The blot was washed to a final stringency of 0.1% SDS and 0.2% Blocking reagent at 60℃ for 20 min. Ethidium bromide-stained rRNA bands as an indicator of equal loading (rRNA). Relative expression intensity was calculated by scanning the developed X-film with a densitometer.

expression, its expression in response to stresses and hormones were investigated in *R. glutinosa* leaves. The expression of *RgCu-ZnSOD1* mRNA was increased most about 20% by wounding and paraquat. However, *RgCu-ZnSOD1* mRNA was

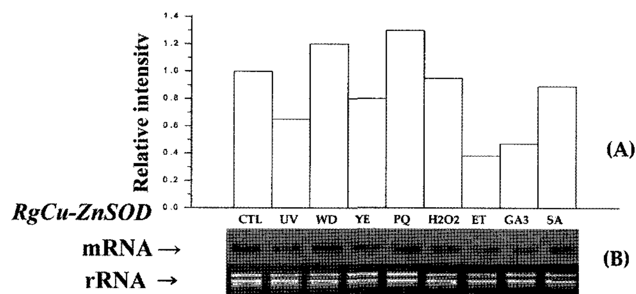

Fig. 6. Differential expression of *RgCu-ZnSOD1* mRNA in response to stresses and phytohormones. A. The histograms show the relative intensity of mRNA levels in the treatments. B. Expression of *RgCu-ZnSOD* mRNA in response to various stresses and stress-related phytohormones. CTL, control; UV, ultraviolet; WD, wounding; YE, yeast extract; PQ, paraquat; H_2O_2, hydrogen peroxide; ET, ethylene and SA, salicylic acid. Twenty micrograms of total RNA were resolved on a 1.0% (w/v) agarose/formaldehyde gel and transferred to a nylon membrane, then hybridized with the alkaline phosphatase labeled *RgCu-ZnSOD* insert DNA. The blot was washed to a final stringency of 0.1% SDS and 0.2% Blocking reagent at 60 ℃ for 20 min. Ethidium bromide-stained rRNA bands as an indicator of equal loading (rRNA). Relative expression intensity was calculated by scanning the developed X-film with a densitometer.

decreased over 50% by ethylene and GA_3 (Fig. 6). The differential regulation of *SOD* gene expression by biotic and abiotic factors is well documented in many plant species. In maize, the chloroplast and cytosolic SODs are increased significantly, however, the mitochondrial form is increased only slightly by paraquat (Matters & Scandalios, 1986). In tomato, the levels of cytosolic and chloroplast *SOD* transcripts increase in response to paraquat and mechanical wounding. The level of the cytosolic transcript and the respective isozyme activity increased dramatically during prolonged drought stress while the chloroplast transcript remained unaffected (Perl-Treves & Galun, 1991). *Cu-ZnSOD* gene expression is also increased by paraquat and mechanical wounding in pine (Karpinska *et al.*, 2001).

Thus, the expression of *RgCu-ZnSOD1* in response to stresses shares both similarities and differences to that of *Cu-ZnSOD*. This result is consistent with recent report on the differential response of SOD activity to paraquat in *R. glutinosa* and soybean. The activity increased in *R. glutinosa* but decreased in soybean in response to paraquat (Choi *et al.*, 2004). The differential responses of the antioxidant enzymes to paraquat are often noticed within and among plant species. Paraquat treatments result in increased *SOD* in maize (Matters & Scandalios, 1986) and wheat seedlings (Okuda *et al.*, 1992). In tobacco, however, the responses of *SOD* gene expression to paraquat are contrasting in the two *Nicotiana* species. The abundance of *Fe-SOD* mRNA is increased in *N. plumbaginifolia* (Tsang *et al.*, 1991), but is decreased in *N. tabacum* in response to paraquat (Kurepa *et al.*, 1997).

Taken together, RgCu-ZnSOD1 shares most structural features conserved among plant cytosolic Cu-ZnSODs. However, its expression responses to stresses and phytohormones showed stress-specific similarities and differences with those of *Cu-ZnSODs* from other plant species. It is not clear whether a significant increase in *RgCu-ZnSOD1*mRNA by paraquat is related to the unusual paraquat tolerance of *R. glutinosa*. Further study, including inhibition of *RgCu-ZnSOD1* expression by RNA interference, will give more direct evidences on its role in paraquat tolerance.

LITERATURE CITED

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