Structure and differential response to abscisic acid of two promoters for the cytosolic copper/zinc-superoxide dismutase genes, *SodCc1* and *SodCc2*, in rice protoplasts

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Abstract **We determined the 5′-flanking sequences of two nuclear genes (*SodCc1* and *SodCc2*) encoding cytosolic copper/zinc-superoxide dismutase in rice (*Oryza sativa* L.). Utilizing transient β-glucuronidase (GUS) reporter assays, functional promoter–GUS analysis was performed in rice protoplasts exposed to the phytohormone abscisic acid (ABA) or the antioxidant sulfhydryl reagent, dithiothreitol (DTT). Transcriptional activities from both *SodCc*–GUS fusions were stimulated by DTT, which induces the promoter activity of the tobacco *SodCc* gene [Proc. Natl. Acad. Sci. USA 90 (1993) 3108–3112]. ABA had no effect on *SodCc1*–GUS expression but specifically induced the gene expression of the *SodCc2*–GUS fusion. The simultaneous application of ABA and gibberellin A_3, however, abolished the enhancing effect of ABA. These results indicated that two rice *SodCc* promoters differentially respond to externally supplied ABA and that one of the regulatory factors for plant *SodCc* expression is ABA in addition to cellular redox-modulating antioxidants.**

Key words: Abscisic acid; Differential gene regulation; Inducible promoter; Superoxide dismutase; Cellular redox; Rice

1. Introduction

Plants are constantly challenged by various types of stress resulting from climatic and other environmental fluctuations, and generate specific gene products that confer resistance and adaptation under severe conditions. One protein intimately associated with stress tolerance is superoxide dismutase (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1) [1]. SOD is a metalloenzyme that promotes the disproportionation of the superoxide anion radical into molecular oxygen and hydrogen peroxide. Cooperating with other enzymes involved in hydrogen peroxide-detoxification systems, SOD contributes to minimizing oxidative damage to plant cells. Three types of SOD are distinguished by their associating metals: copper/zinc- (Cu/Zn), manganese- (Mn-), and iron- (Fe-) isozymes [2]. Cu/Zn- and Mn-metalloenzymes are ubiquitously distributed among plants. Cu/Zn-SOD is the most abundant, occurring as two distinct isoforms that are targeted to the cytosol and the chloroplast stroma. Mn-SOD typically appears in the mitochondrial matrix, whereas Fe-SOD is found within the plastids of limited plant species. SOD activities in plants are not only controlled in a developmental manner, but they are also differentially induced by a number of environmental cues which can cause oxidative stress in living cells (for a review, see [1]). Each cDNA for three plant SOD genes (*Sod*) has been cloned and sequenced from a variety of plant species. Because of its potential importance, cloned cDNAs have been used in transgenic studies to achieve the enhancement of stress tolerance in plants (for a review, see [3]). Gel blot analyses of RNA using isolated cDNAs as probes have shown that the differential and developmental regulation of *Sod* expression primarily operates at the transcript level [4,5]. While studies have extensively focused upon the isolation of cDNA clones, genetic engineering for antioxidative protection and analyses of the steady state-transcript levels, characterization of the *Sod* genes [6–9] and functional promoter studies [9–11] have just begun. Recently, the transcriptional regulation of two plant *Sod* promoters has been investigated in transgenic tobacco. Hérouart et al. [10] have reported that reduced forms of sulfhydryl antioxidants, such as glutathione and dithiothreitol (DTT), can stimulate the transcriptional activity of the tobacco *SodCc* promoter, suggesting the modulation of the *SodCc* expression by cellular redox levels. The *SodCc* promoter also directs developmentally regulated gene expression [11]. Using a short promoter segment, Kardish et al. [9] have shown the spatial and light-responsive regulation of the tomato chloroplastic Cu/Zn-SOD gene.

To gain a better understanding of the regulation of plant *Sod* expression, we set out to characterize individual members of *Sod* families in rice (*Oryza sativa* L.) plants. We isolated cDNA clones coding for Cu/Zn- and Mn-isoproteins from developing seeds [12,13]. We also characterized two rice genes (*SodCc1* and *SodCc2*)[1] encoding cytosolic Cu/Zn-SOD, which brought the first information on the *Sod* exon/intron organization from plant sources [6,7]. As one approach to define the molecular mechanisms responsible for plant *Sod* regulation and to iden-

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Abbreviations: ABA, abscisic acid; ABRE, abscisic acid-responsive element; Cu/Zn-SOD, copper/zinc-superoxide dismutase; GA_3, gibberellin A_3; GUS, β-glucuronidase; SOD, superoxide dismutase; *Sod*, superoxide dismutase gene; *SodCc2*, cytosolic copper/zinc-superoxide dismutase gene.

[1] Two rice cytosolic Cu/Zn-SOD genes, *SodCc1* and *SodCc2*, were previously called *sodA* and *sodB*, respectively, but have been renamed in accordance with the recommended nomenclature of plant genes (Zilinskas, B.A., Asada, K., Galun, E., Inzé, D. and Tanaka, K. (1994) Plant Mol. Biol. Rep. 12, S73–S74).

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tify the actual inducers of the gene expression, we constructed two chimeric β-glucuronidase (GUS) reporter genes under the transcriptional control of the 5′ regions of *SodCc1* and *SodCc2* genes. Here, we investigated the structures of the 5′-flanking sequences of rice *SodCc* genes and examined the effects of the phytohormone, abscisic acid (ABA), on the transcriptional activities of the *SodCc* promoters in rice protoplasts. The study provided evidence that the two *SodCc* promoters differentially respond to ABA, and that the 5′ region of the *SodCc2* gene conferred enhanced reporter gene expression in a transient GUS assay. This is the first demonstration of the involvement of ABA in the regulation of plant *Sod* expression by a functional promoter analysis.

2. Materials and methods

2.1. Nucleotide sequence determination of rice SodCc 5′-flanking regions

The rice *SodCc* genes have been isolated and characterized [6,7]. Successively deleted plasmids were generated by means of exonuclease digestion after each 5′-flanking sequence was subcloned into the Bluescript vector (Stratagene, La Jolla, CA). Double-stranded DNA templates were sequenced in both directions using the ABI373A sequenator (Applied Biosystems, Foster City, CA).

2.2. Construction of the SodCc–GUS fusion genes

Two translational fusions between *SodCc* and GUS genes (pSodCc1-GUS1 and pSodCc2-GUS1) were constructed in the transient GUS expression vector pBI221 [14]. To construct pSodCc1-GUS1, site-directed mutagenesis was performed by means of polymerase chain reaction (PCR). A 3 kb *Sac*I–*Nco*I fragment which corresponds to the 5′ region of the *SodCc1* gene was recloned into Bluescript from the genomic λ clone gSOD27 (the *Sac*I site is shown in Fig. 1A and the *Nco*I site is located in the middle of the third exon of the gene). PCR was performed using the plasmid subclone as a template, the M13 primer M4 (Takara Shuzo, Otsu, Japan), and the mutated antisense oligonucleotide primer (5′-CTCACTGCTAGCAAGCACA-3′; the *Nhe*I site is underlined) to introduce an *Nhe*I site in the second exon of the *SodCc1* gene. The *Nhe*I site was placed at the position identical to that found in the *SodCc2* gene. An amplified 2.3 kb fragment was first cloned into the pGEM-T vector (Promega, Madison, WI). Following *Pst*I (in the polylinker) and *Nhe*I digestion, the excised *SodCc1* 5′ fragment was inserted between the *Pst*I and *Xba*I sites in pBI221 (*Nhe*I and *Xba*I produce compatible 5′ overhangs). Plasmid pSodCc2-GUS1 was constructed as follows. A 7 kb-DNA fragment encompassing the entire *SodCc2* gene and its flanking regions was obtained by *Bam*HI digestion of gSOD7 λDNA and integrated into Bluescript at the appropriate restriction site. Following digestion at the *Hin*dIII site in the polylinker and at the *Nhe*I site located in the second exon, the released 2.2 kb fragment corresponding to the *SodCc2* 5′ region was fused translationally to the coding sequence of the GUS gene by ligation with the *Hin*dIII/*Xba*I-cut pBI221. Both fusion constructs contain the 5′-flanking sequence, the 5′ non-coding exon/intron, and the coding sequence for up to 9 NH_2-terminal amino acids of the corresponding SOD polypeptide instead of the cauliflower mosaic virus (CaMV) 35S RNA promoter in the original construct (Fig. 2). Translational fusion junctions in these chimeric constructs were verified by supercoil sequencing.

2.3. Introduction of fusion genes into rice protoplasts

Fusion plasmids were introduced into rice protoplasts according to Li et al. [15] with slight modifications. Protoplasts were released from suspension cultured cells derived from embryogenic calli (*Oryza sativa*

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SacI
GAGCTCATCA GAGATGTTTA TGTGTCGGTC CTTATATAAA TCTATCATAG ATGAGTTCTT -2196
ACATGTGGCT AGTCATTTCC TTGAGCTTCA TTACAACGGG ATTTAGTTTA ACTCGTGTCA -2136
GAGACAAATT TTTTTGGTCC ATCGTAGATA GACCGTCATA GATGGATTGG TCTGGCGCAA -2076
TGAATTGTGT TGCTTGATCT GGATCTATTG GTCAATTAAA ATGAAGTGCT CCCAGCTGAG -2016
                                  EcoRV
TTTAATTCTT CGGGTGTGGT CGGTGAGTAT GCCAGATATC CCTTTCACAC ACACTGCTTG -1956
CAGCAGCATT AGTGTCAACA AAACGTGGTT GGAAATCCGT CAAAAAAAAA AACGTGGTTG -1896
GAAATTGCCG GTCCTTTCAG AATCGGTGTA CACACAAGAC GATGGTACTC GCTCGGGAGA -1836
GTAATAACGT AAGAGGGTTA GATGGCAGTA TTACGGCCAT ATTCAGTAGA GCATAAATTG -1776
TAAATTCAAC GATGAAGATT ATTATACGAG TAGTATTTGA AGCCTAATCT TCAAGCTCAT -1716
CTATCGAAAA TTATTGCCGT GCTTCTAAAA ATGAAATTTC TTCCCCGAAA AAAATATTCT -1656
TAAAACACTT TATTCTACCA TATTATTTAT AGTACTACTC TCCCTCCGAC AAACATTAGA -1596
TGTCCAAAAA CATCAAAAAA GTCTTTTCAT CAGAGGTTAT TTTTCAGTAT TAACATTTAA -1536
ATCGTTAAGA ATACACAACA CCGTCGTTTC TCTTATTCGG GAAAAAAGCG AAACAACATA -1476
TTTACAAACG AAAAATAATT TATAAATAAA ACTTTTATAT ATGTGTTTTT AGCGATCTAA -1416
ATCCAAAGAT GAAAAAAAAA CTTCGATCAA AAAAATCTTA AAATAAACTA TAATTTAAGA -1356
TTGAAAATTT CAAAATTTGG TTGTGATAAA CATGAGCGAA AAGATGAGAC CCCAAATTTT -1296
                                  ApaI
TTTTAATTAA ATTATTATTA TAATAAATAA ACGAGGGCCC CCAATGTTGG CTGGTGGCTG -1236
CAACTGCAAG CTGCCAGTTT GACTACAAAT ACCACCGCAC ACCGCTGGAG GGAGGGAAC -1176
CTTCCAGAAG CTCCAGATTC CAAACCAGCA GGAGTCGCCT CGCCTCCTCC TTCATCCTCC -1116
        SacII
TCGTCGTCGC CGCGGGGGTC GCCTGAGgta ggaatcccaa caacccccc ccccccccc -1056
          HindIII
cacccaagca agcttctagg gttttcgtga cttgtttcct ccggtttcgt gggaggcggt  -996
gggtctccct ccctccctgc gtgtggatct cagatggatc ggtgtttcct tgttggtctt  -936
gctgcttcgc ggggatttgg ggcttcgggc gggctggggg tggggggggg gattgttgtg  -876
gatgttgatt gatcgatcgg tgttgttggt tcgggggcgat tggttggttg gtgggggagga -816
acggctcgtc tcgtgtgttt gaatccatat gcaagttcaa gtgatgcgag agtgttttga  -756
tgcgtggacc tgctgcttcc ttttttttct ttcttttga gcgatgattc gtgctgctgc  -696
ggagggatac ggatttgtta gttgccgatt actagtaata atcattggaa ttgtgtactg  -636
ggctctggct gctcatgatg cgatgggctt tgtgatatgt tcatgattag atttagacat  -576
gtttttttctg caagtgtgga gttgattgca gagtgtcctt ccctgatatg tcgggggatt  -516
gggtggatgt agagaattgt agatttggga gttattggac tagccaacag acagtttcct  -456
   EcoRI
cgtgaattcg atcccttcat gcagaaatga atggagggat aggatgaaat ggggtgagaa  -396
gaatggatag ctaagattga aacttaccct ttagatgggt gggaaatcat atcccaaacc  -336
gattttctag ggataattcc tctatactag acgtgtgttt tgtttgtatc tagtatgttt  -276
gcagtgggca gtggcatttg ttctatgatg cctttggttt acatgctttg gaaaaaacaa  -216
tagatcattg ggccatattg ttatctttttt ctgactagta gtaaatgatg cgtctgtgtt  -156
gcttctgctt taacatcttt tcaatcttca ctaggataat ttcatagttc aatatgttct   -96
                          XbaI
ttgggaactt ggttgggttt taggacctct agaggctact tctgccactt gctaagtaac   -36
tgttttgtgg aatttgttta cagATCACAT TAACAATGGT GAAGGCTGTT GTTGTGCTTG   25
GTAGCAGTGA GATTGTTAAG GGCACTATCC ACTTTGTCCA AGAGGGAGAT Ggtatgccat   85
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B

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BamHI
GGATCCTGAC TTCAGCAAGT TTGGTTAGGA TTTCTGATGA TGAAAACAGG ATTAATGATA -2069
ACTTGAAGGT TGGTATTGTA TTGTCCTCCT TGAGCTTATT CATTTGAGAT ACCGGTAAAT -2009
          PstI
CCTCATATTA CTTCTGCAGA TTGTTGTACA CCTTCCAAAC AAACAACAAA CTGTAGGGAC -1949
ATTGCAACAT TATCATCTGC ATTACAATGT TGCTATTGTC AGCATCAAAG GTTTCCGTTG -1889
TCTTCGTACG GAAGAATTTC ATGATCCAGG GCAAATTAAG TGTAAGAAGG TATTATCTGT -1829
GGGACGTGTC TTCAAGTCAG GCAAATTAAT GGCCACAGGT GGGATATTGA CGGACAAACC -1769
AAGCAAACTT GACTGCAAAG AACTTATGGT CTCCACTTGT AGAATCACTA AGGTACAGTG -1709
AATCTCCAAA TTGAAGTCTT GCTTTTCCTC GCAAAAGAAA GGCTTTAATT TTCTTATATT -1649
GGAAATAATT TGTTGCCTTA CAATATGAAC AAGAGCAATT TAATATAACA CTACCAAACA -1589
                                  ApaI
TTGCCATGTC TGCTTACCAA ACCTAGCCAA AGGGCCCGTC TAATAAATTG ATGGCCATAC -1529
CTTAGGCAAG ATTTGGGAAA TAAATGAGTG CATGACATAT GTTTCCTAGG ATTAAAAGAA -1469
CGGAAATGAA ACAACTGTTT TACGAAGTGT GGAAAGATTG GCAAAGTGTG AAACGCTCCT -1409
                                                  ApaI
GCTTCTGCGG AACTACGTGT GTAATCCTTT GTTTTTCTGC TAGGCTGGGA TTGGAGGGCC -1349
CCTGATTGAT TTTGATGGGA ATTTCGTTGG TATGGTAGGA AGGAGACTCC GTATTTACCA -1289
AGGGATACAA TTCTGAAATT ATTGAGCTAT TTTGATGGAG AAGGGTATGT CTTCTATGTT -1229
TATATGTATA CCTGTGTTTT CAACTTTTCT ATGTCTTGTT ATTTAGTTTC CTTCAAATTT -1169
TCAACTTTTC TGTGTCTTGT TGTAGGGATG TCTCTGCTGA GATCATGGAC AGAAACAACA -1109
GATGCCCTGT GCCCAAGATA CGTTGGCATT ATCCTCACTT CTGCAAGCCA CGCAAGTCAG -1049
AGAAGTGTGG TTAATATGAA AAGTACTAGA TGAGACCCCT GCGTGTTGCA GCGGGAAATC  -989
TTGTGTATAA TATTAAAATT TGTAAATTGA TTATGGTAAA CATATTAGTA AAATATTGAA  -929
ACTGATTAAC AAAGGAACTT GATTGCTATC ATACAAAAGA CTGACCAATC GAACGAAAGA  -869
GTCATGCTCC CTAAGACATT TTGAATGGGC CTAGCCATCA GATTTTGATC TAACAGACAG  -809
                                        EcoRV
GGAGTCTTAT CGGAGAGGGA GTAGGTAACC GAGATATCTT CTGATGAGAA TAAGCAAATC  -749
CCTATTTTTT TTGTTGAAGA AAAGAAAAGC GTGTCCACGT GGACTGGACT CTGAAATTTT  -689
CCTCCTCCCT TCTGGAGTCT TCCTCATCAG AAATCAGAAG AGGAGAGGGT GGGCAACTCG  -629
CAGATCGCCT TCTCGTCGCG CTCGCGCCGC AGGGGTCGCC TGAGgtatgc agcttcacct  -569
ccccccaactt tctagggttc taatcgcctc tgctcgctcg gattatgcgt ggtgggtatg  -509
gctccgattc cgccggagta gctggatctg tgtgccccgt gctaatttag gtttggcttt  -449
cgattcggcc gcgcttcggt ttgttctcgc gcgtgattgc ttcgttcggc catagggctc  -389
atctcaggct cgacatgtgg acggccacaa acataaaaaa tccttgttaa atttacggtt  -329
catgtggtag gggggctttt caggatcgga ggtttaggtg atttggagta gcaaaacgat  -269
          EcoRI
tttgctgtgt tagaggaattc gagaatctgt gccgtcagat gctaattagc tggttttgaa  -209
caaatgttaa gacatcctga tattatttgc tgcgtttaat gttcaaactt tttgcacggg  -149
tgttgctgag ctataaagga tatgaggata tagtaccaag ttctatcgtt ttgttctgat   -89
acatacaatt tgctgtagtt tatggcacca tcatactgag atatatatac ctgtgcacct   -29
                                        NheI
ttcggcttct gtgcagAACA CATAGACAAT GGTGAAGGCT GTTGCTGTGC TTGCTAGCAG    32
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Fig. 1. Nucleotide sequences of the 5′ regions of rice *SodCc* genes. (A) The *SodCc1* gene. (B) The *SodCc2* gene. Nucleotide residues are numbered relative to translation start site as +1. Bold-face indicates presumptive CAAT boxes, the 5′ end of the longest cDNA and the translation start-methionine codon. The 5′ non-coding intron is shown in lower letter case. Motifs similar to the heat shock elements (GAANNTTC) are underlined. Direct repeat sequences are shown in italic letters. A complementary sequence to the mutated oligonucleotide primer used for pSodCc1-GUS1 construction is also indicated in italics (Fig. 1A). The double underline refers to the putative ABA-responsive element (ACGTG) or the as-1 motif (TGACG). The dotted line indicates the restriction site with its name above the sequence.

L. cv. Nipponbare) during a 3-hour incubation at 28°C without shaking in enzyme solution containing 4% Cellulase RS, 1% Macerozyme R-10 (both from Yakult, Tokyo, Japan) and 0.4 M mannitol. The protoplast suspension was filtered through a 30 μm sieve to remove undigested cell clumps and debris, then mixed with a two volumes of KMC solution [15]. The filtrates were centrifuged at $130 \times g$ for 5 min and protoplast pellets were washed twice with KMC. Protoplasts were resuspended (4×10^6 protoplasts/ml) in MaMg solution [15]. Aliquots (1 ml) of protoplast suspension were mixed with plasmid DNA and carrier DNA (each 40 μg/ml). After incubating protoplast/DNA mixtures for 15 min at room temperature, 1.85 ml of 40% polyethyleneglycol (PEG) 6,000 in MaMg was gently applied. After an incubation for 30 min at room temperature, protoplasts were suspended in 40 ml KMC and centrifuged at $130 \times g$ for 5 min. Protoplast pellets were resuspended in General medium [15] supplement with sucrose (106.9 g per liter) and cultured at 28°C for 48 h in the dark.

2.4. Assay for transient GUS activity

To suppress endogenous GUS activity, a modified protocol [16] was employed for the fluorimetric determination of 4-methylumbelliferone (4-MU) produced from the cognate glucuronide (4-methylumbelliferyl-β-D-glucuronide) in protoplast extracts. The protein concentration was measured by the method of Bradford [17]. GUS activity was normalized according to the expression derived from pBI221 in control experiments (average 204 pmol of 4-MU produced per minute per μg of protein obtained from four independent experiments = 1).

3. Results

3.1. Characterization of 5′-flanking regions of rice SodCc*genes*

We determined the nucleotide sequences of more than 1-kilobase at the 5′-flanking ends of two *SodCc* genes from rice (Fig. 1). The 5′ upstream regions of these genes contained sequences with homology to known regulatory elements identified in other plant genes. Neither upstream region displayed consensus TATA signals, but both had a CAAT box. Two and three putative ABA-responsive elements (ABREs) [18,19] were found in the *SodCc1* and *SodCc2* 5′ regions, respectively. Another plant *cis*-acting factor, as-1 [20], was found in the *SodCc2* promoter, but not in the *SodCc1* region. Several putative heat-shock elements [21] were found in each promoter region (*SodCc1*, 7; *SodCc2*, 4). Complete direct repeats, consisting of 17 and 24 bp-units, were identified in *SodCc1* and *SodCc2* 5′ upstream sequences, respectively. No nucleotide similarity was found between repeated motifs. ABREs in the *SodCc1* 5′ region appeared within the direct repeats. Although the structural organization was quite similar between the two genes [6,7], the 5′ upstream regions had only limited sequence homology, except for a stretch of 38 bp between positions −1360 and −1323 bp in the *SodCc1* gene and between −981 and −946 bp in the *SodCc2* gene (Fig. 2A).

3.2. Transcriptional activities of rice the SodCc *promoters in protoplasts*

To analyze *SodCc* promoter functions, the expression of chimeric genes containing 5′-flanking regions of rice *SodCc1* and *SodCc2* genes linked to the coding sequence of GUS was examined in protoplasts prepared from suspension cultured cells after PEG-mediated DNA uptake. Fusion constructs between rice *SodCc* 5′ regions and the GUS gene are shown in Fig. 3. Translational fusion allowed the inclusion of the first intron, which occurred in the 5′ untranslated region of each *SodCc* gene, in the chimeric constructs. Fig. 4 shows the results of transient GUS assays for rice protoplasts transformed with pBI221 and two *SodCc*–GUS fusions. Since the CaMV 35S promoter confers constitutive expression to a downstream reporter gene [22], pBI221 was used as the positive control plasmid of PEG-mediated transformation and also for the negative standard of inducible or responsive gene expression. As shown in Fig. 4A, the application of ABA and DTT to transformed protoplasts did not substantially alter the pattern of GUS activities from this constitutive expression cassette. Basal levels of GUS expression from pSodCc1-GUS1 and pSodCc2-GUS1 were 1.6- and 0.6-fold, respectively, compared to that of pBI221. Expression of both *SodCc*–GUS constructs was stimulated by DTT (1 mM), a known inducer of tobacco *SodCc* transcription [10]. Since both rice *SodCc*-promoter sequences contained ABRE motifs, we added ABA (100 μM) for the transient assay. The GUS activity derived from pSodCc1-GUS1 remained at the same level as those of controls, and therefore the *SodCc1* promoter did not respond to ABA. However, exogenous ABA enhanced the reporter gene expression from pSodCc2-GUS1 with the average increase in enzyme activity being about 7-fold. This was in marked contrast to the

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                          ** * * * * ***  *****    *  * *  **
SodCc2 (rice)      -981 TAATATT-AAAATTTGTAAATT-GATTATGGTAAACAT  -946
                        ||| ||| |||||||  ||||| | || || |||||||
SodCc1 (rice)     -1360 TAAGATTGAAAATTTCAAAATTTGGTTGTGATAAACAT -1323
                         || | | | | ||||||||||  | |  | |  ||
SodCc (tobacco)    -423 CAAAAATAATATTTTCAAAATTAAGGTAAGGTGCACGC  -386
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B

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SodCc1   -1491 AAGCGAAACAACATATTTACAAACGAAAAATAATTTATAAATAAAACTTTTATATA-TGTGTTTTTAGCGATCTAAAT -1414
               ||||||||| |||||||||| ||| |||||||||| |||||||||||||||||| ||||     ||||||||||||
Trxh      -455 AAGCGAAACGACATATTTACGAACACAAAATAATTTGTAAATAAAACTTTTATATACTGTG-----AGCGATCTAAAT  -383
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Fig. 2. Homologous sequences in the 5′-flanking regions between rice *SodCc* and other genes. The vertical bars represent nucleotide identity and the minus signs show gaps. Numbers refer to nucleotide positions relative to start of translation (*SodCc1* and *SodCc2*) or transcription (tobacco *SodCc* and rice thioredoxin h) as +1. (A) Homology among rice *SodCc1*, *SodCc2* and tobacco *SodCc* genes. Conserved residues are marked with asterisks above the alignment. (B) Homology in the 5′ upstream regions between *SodCc1* and thioredoxin h (*Trxh*) genes from rice plants. The genomic sequence for rice thioredoxin h was cited from the GenBank database (D26547 deposited by Y. Ishiwatari and M. Chino).

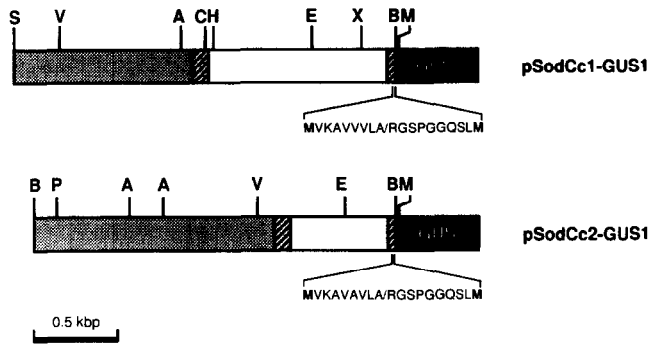

Fig. 3. Two *SodCc*–GUS chimeric constructs (pSodCc1-GUS1 and pSodCc2-GUS1) used for transient transformation of rice protoplasts. Hatched and open boxes refer to exons and introns, respectively, while shaded boxes indicate the 5′-flanking regions. The GUS coding sequence shown by a closed box is not represented in scale. The amino acid sequence of a translational fusion between *SodCc* and GUS genes is indicated in one letter code below each construct. Boldface residues denote *SodCc* and GUS translation initiation methionines and fusion junctions are indicated by slashes. Restriction sites are: A, *Apa*I; B, *Bam*HI; C, *Sac*II; E, *Eco*RI; H, *Hin*dIII; M, *Sma*I; P, *Pst*I; S, *Sac*I; V, *Eco*RV; X, *Xba*I.

results from pBI221 and pSodCc1-GUS1, in which there was no apparent increase in GUS activity in the presence of ABA. Since ABA and gibberellin A_3(GA_3) often function as antagonistic regulators, we examined whether the effect of ABA in the *SodCc2*–GUS expression is hampered by GA_3. Incubation in the presence of both phytohormones, indeed, resulted in no enhancement of the activity, thereby suggesting that the transcriptional activity is regulated by both phytohormones. The ABA concentrations (0, 1, 5, 10 and 100 μM) were titrated to induce the full activity of the *SodCc2* promoter. Fig. 4B shows that 10 μM ABA is sufficient to maximally induce the *SodCc2*–GUS expression. The expression of GUS activities was proportional to ABA concentration from 1 to 10 μM. These results clearly demonstrated that hormonal regulation of the expression of the *SodCc2*–GUS chimeric gene is specific to the 5′ region of the *SodCc2* gene. We therefore concluded that the 2.2 kb fragment from the *SodCc2* 5′ region at least partly comprised the *cis*-acting element(s) necessary for ABA-mediated hormonal regulation.

4. Discussion

To identify the regulatory factors of plant *Sod* families, we determined the nucleotide sequences of the 5′-flanking regions of two *SodCc* genes from rice and investigated the transient transcriptional activities of the promoters in a homologous gene expression system. Both *SodCc* promoters were activated by exogenous DTT, but the responsiveness of the promoters to ABA was markedly different. The *SodCc2* promoter was stimulated in response to ABA, but the *SodCc1* promoter was not. This differential expression of two similar genes indicates that they play somewhat different roles in the stress response and that different mechanisms occur in the control of the *SodCc* expression in rice under some environmental conditions. Also, our results confirmed the activation of plant *SodCc* promoters by antioxidant sulfhydryl molecules, suggesting that the cellular redox-mediated regulation is a common regulatory aspect of the *SodCc* expression in plants.

Phytohormones act positively upon plant *Sod* expression at steady state mRNA levels. Ethylene and salicylic acid stimulate Mn-SOD gene expression in tobacco and rubber trees [8,23]. Ethylene also induces the accumulation of tomato *SodCc* transcripts [4]. Our results showed that ABA promotes the reporter gene expression driven by the 5′ region of an rice *SodCc* gene, providing direct evidence that this phytohormone is involved in the transcriptional activation of the plant *Sod* promoter. Kanematsu and Asada commented [24] that rice cytosolic Cu/Zn-SODs III and IV, either of which is the putative *SodCc2* gene product [12], are dominant in the seed embryos but their activities decrease following seed germination. The pattern of transient *SodCc2*–GUS expression correlated well with this

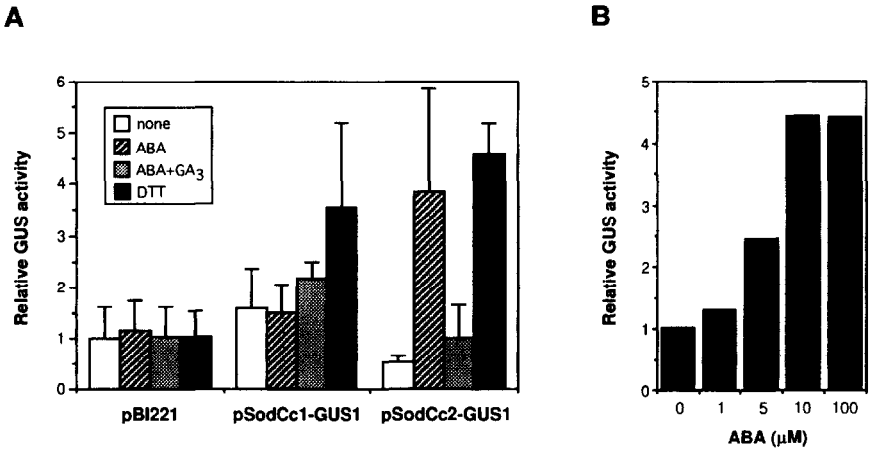

Fig. 4. Transient GUS expression in rice protoplasts transformed with chimeric constructs. (A) The effects of ABA and DTT on transient GUS expression from pBI221, pSodCc1-GUS1 and pSodCc2-GUS1. Transformed rice protoplasts were incubated at 28°C for 48 h without or with the following at the indicated concentrations: ABA, 100 μM; GA_3, 100 μM; DTT, 1 mM. Thereafter, GUS activities (pmol 4-MU produced per minute per microgram protein) were fluorimetrically determined and normalized by comparison with control experiments of pBI221. Mean values from four independent experiments are shown. Vertical lines refer to standard errors. (B) The effect of various concentrations of ABA on GUS activity from pSodCc2-GUS1. Protoplasts were incubated with the indicated concentrations of ABA for 48 h immediately after transformation with pSodCc2-GUS. The relative GUS activity is shown from two independent experiments. The results are independent from those shown in Fig. 4A, so the degree of induction by DTT at 100 μM is somewhat altered.

activity profile since the transcriptional activity of the *SodCc2* 5′ region was elevated by ABA , but the effect was abolished by the germination-promotive GA_3. ABA differentially activates the expression of other antioxidant defense multigenes encoding catalase in maize endosperms [25]. These observations imply that specific member(s) of antioxidant defense multigene families such as those of *Sod* and *Cat* are organized in an ABA-regulated manner in the plant genome and that their expression is coordinately conducted in response to increased ABA levels within plant cells during seed maturation or under environmental stresses such as desiccation, high osmotic pressure and low temperature.

We found several putative *cis*-acting elements which are supposed to be involved in the regulation of rice *SodCc* genes. Each promoter contained several heat shock-resemble elements. Tobacco *SodCc* expression is induced by heat shock and there are several heat shock elements in its promoter region [5,11]. Motifs like ABREs have been identified in both rice *SodCc* promoters, but the elements in the *SodCc1* sequence seemed not to function since this promoter was not responsive to ABA. One of the ABRE (positions −716 to −705) in the *SodCc2* promoter is of particular interest. The motif and surrounding nucleotides form a perfect 12-bp palindromic structure (5′-GTCCACGTGGAC-3′) with the G-box core, CACGTG, in its center. The hexameric sequence is classified into the A type G-box according to the sequence analogy of the flanking regions [26]. Hérouart et al. [11] pointed out the presence of two homologous boxes between the promoters for the tobacco *SodCc* and the bean chalcone synthase genes. Apparent homology to these sequences, however, was not detected in the rice promoters. Instead, the tobacco *SodCc* sequence showed moderate homology to the conserved region between two rice promoters (Fig. 2A). Whether or not the region plays a role in plant *SodCc* regulation remains to be proven, but it might be responsible for coordinated induction by thiol molecules. A search for sequence similarities of rice *SodCc* 5′-flanking sequences in the GenBank database revealed the striking homology of over 70-nucleotide residues in the promoter sequences of the *SodCc1* and rice thioredoxin h genes (GenBank Accession Number D26547) (Fig. 2B). This long stretch of nucleotide similarity was not found in the *SodCc2* promoter. Thioredoxin h is a cytosolic form of the protein in higher plant cells and it participates in a number of cellular redox reactions where disulfide linkages formed as a consequence of oxidative damage may be reversibly reduced to a dithiol. Although the significance of the conserved sequence in the regulation of the *SodCc1* gene is yet to be tested, the expression patterns of the *SodCc1* and the thioredoxin h genes in plants should be examined since the state of cellular redox appears to involve the regulation of plant *SodCc* genes.

In conclusion, we examined the structure and function of two promoters of the rice *SodCc* genes in rice protoplasts by transient assays for GUS activity after transformation with promoter-reporter gene fusions. Both promoter activities were coordinately induced by an antioxidant sulfhydryl reagent but their responses to ABA markedly differed. Dissecting *cis*-acting regulatory sequences involved in sulfhydryl reagent- and ABA-responses and characterization of *trans*-acting factors associated with each promoter region should provide more insight into the mechanisms of plant *Sod* expression.

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