

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 A₃, 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 mini-

mizing 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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The nucleotide sequence data reported in this paper have been deposited in the GenBank/EMBL/DBJ Nucleotide Sequence Databases under the accession numbers L19434 (*SodCc2*) and L19435 (*SodCc1*).

Abbreviations: ABA, abscisic acid; ABRE, abscisic acid-responsive element; Cu/Zn-SOD, copper/zinc-superoxide dismutase; GA₃, gibberellin A₃; GUS, β -glucuronidase; SOD, superoxide dismutase; *Sod*, superoxide dismutase gene; *SodCc2*, cytosolic copper/zinc-superoxide dismutase gene.

¹Two 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).

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 (p*SodCc1*-GUS1 and p*SodCc2*-GUS1) were constructed in the transient GUS expression vector pBI221 [14]. To construct p*SodCc1*-GUS1, site-directed mutagenesis was performed by means of polymerase chain reac-

tion (PCR). A 3 kb *SacI*-*NcoI* fragment which corresponds to the 5' region of the *SodCc1* gene was recloned into Bluescript from the genomic λ clone gSOD27 (the *SacI* site is shown in Fig. 1A and the *NcoI* 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 *NheI* site is underlined) to introduce an *NheI* site in the second exon of the *SodCc1* gene. The *NheI* 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 *PstI* (in the polylinker) and *NheI* digestion, the excised *SodCc1* 5' fragment was inserted between the *PstI* and *XbaI* sites in pBI221 (*NheI* and *XbaI* produce compatible 5' overhangs). Plasmid p*SodCc2*-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 λ DNA and integrated into Bluescript at the appropriate restriction site. Following digestion at the *Hind*III site in the polylinker and at the *NheI* 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 *Hind*III/*XbaI*-cut pBI221. Both fusion constructs contain the 5'-flanking sequence, the 5' non-coding exon/intron, and the coding sequence for up to 9 NH₂-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*

A	SacI	GAGCTCATCA	GAGATGTTTA	TGTGTCGGTC	CTTATATAAA	TCTATCATAG	ATGAGTTCCT	-2196
		ACATGTGGCT	AGTCATTTCC	TTGAGCTTCA	TTACAACGGG	ATTAGTTTGA	ACTCGTGTCA	-2136
		GAGACAATTT	TTTTTGGTCC	ATCGTAGATA	GACCGTCATA	GATGGATTGG	TCTGGCGCAA	-2076
		TGAAITTTGT	TGCTTGATCT	GGATCTATTG	GTCATTAATA	ATGAAGTGCT	CCCAGCTGAG	-2016
					EcoRV			
		TTTAATTTCT	CGGGTGTGTT	CGGTGAGTAT	GCCAGATATC	CCCTTCACAC	ACACTGCTTG	-1956
		CAGCAGCATT	AGTGTCAACA	AAACGTGGTT	GGAAATCCGT	CAAAAAAATA	AACGTGGTTG	-1896
		GAAATGCGCC	GTCTCTTCAG	AATCGGTGTA	CACACAACAA	GATGGTCTAG	GCTCGGGAGA	-1836
		GTAATAACGT	AAGAGGGTTA	GATGGCAGTA	TTACGGCCAT	ATTCACTAGA	GCATAAAATT	-1776
		TAATATTCAC	GATGAGGATT	ATTATACGAG	TAGTATTGTA	AGGCTAATCT	TCAAGCTCAT	-1716
		CTAATGABAA	TTATTTGCCGT	GCTTCTAATA	ATGAAATTTCT	TTCCCGCAAA	AAAATATTCT	-1656
		TAAACACATT	TATTTACACA	TATTTATTAT	AGTACTACTC	TCCCTCCGAC	AAACATTAGA	-1596
		TGTCCAAAAA	CATCAAAAAA	GCITTTTCAT	CAGAGGTTAT	TTTTCAGTAT	TAACATTITAA	-1536
		ATCGTTAAGA	ATACACAACA	CCGTCTTTTC	TCTTATTCCG	GAAAAAAGCG	AAACAACATA	-1476
		TTTACAAACG	AAAAATAATT	TATAAATAAA	ACTTTTATAT	ATGTGTTTTT	AGCGAICTAA	-1416
		ATGCAAAAGT	GAATAAAAAA	TTTCATGATA	AAAAATCTTA	AAATAAATA	TAATTTAAGA	-1356
		TTGGAATAAT	CAAAAATTTG	TTTCATGATA	CATGACGCTA	AAGATAGACT	CCCAAAATTTT	-1296
					Apal			
		TTTAAATATA	ATTATTATTA	TAATAAATAA	ACGAGGGGCC	CCAATGTGGG	CTGGTGGCTG	-1236
		CAACTGCAAG	CTGCCAGTTT	GACTACAATAT	ACCACCCGAC	ACCGCTGGAG	GGAGGGGAAC	-1176
		CTTC	CAGAAG	CTCCAGATTC	CAAAACAGCA	GGAGTCGCC	CGCCTCCTCC	-1116
		SacII	TGCTGCTGCT	CGCGGGGGTC	GCCTGAGGta	ggaatcccaa	caaccccccc	-1056
		HindIII	caccacaagc	agcgtcttag	gttttcgtga	ctgttttcc	cgggtttcgt	-996
		gggtgttcc	ccctccctgc	gtgtggatct	cagatggatc	ggtgtttcc	tgtttgttgc	-936
		gctgtctcgc	ggggatttgg	ggcttcgggc	gggctggggg	tggggggggg	gattgtttgt	-876
		gatgtttgatt	gatcgatcgg	tgttgttgg	tcggggcgat	tgttgggtg	gtggggagga	-816
		acggctcgct	tcgtgtgttt	gaatccaat	gcaagtccaa	gtgatcgag	agtgtttga	-756
		tgctgtggacc	tgtgtcttcc	tttttttttt	ttctctttga	cgatgatgc	gtgctgtcgc	-696
		ggaggggatac	ggatttggta	gttgcggatt	actgatgaat	atcattggaa	tgttgtactgc	-636
		ggctctcgct	gctcatgatc	cgatgggctt	tgtgatagtc	tcattgatac	atttagaacat	-576
		gttttttctg	caagtgtgga	gttggatgca	gagtgatcct	cctgatagtc	tcaggggatt	-516
		gggtggatgt	agagaattgt	agatttggga	gtatttggac	tagccacaag	acagtttcc	-456
		EcoRI	cgtgaattcg	atcccttcac	gcagaaatga	atggagggat	aggatgaaat	-396
		gaatggatag	ctaagattgc	aacttaccct	ttagatgggt	gggaatcat	atcccaaac	-336
		gattttctag	gataaattcc	tctataactag	acgtgtgttt	tgtttgtcat	tatgtatctt	-276
		gcagtgaggca	gtggcatttg	tctatagatg	cctttgtgtt	acatgctttg	gaaataacaa	-216
		tagatcattg	ggcatatttg	ttatcttttt	ctgactagta	gtaaatgctg	cgtctgtgtt	-156
		gcttctgctt	taaatctctt	tcaatcttca	ctaggataat	tctatagttc	aatatgttct	-96
				XbaI				
		ttgggaacct	ggttgggttt	taggaacctg	agaggctact	tctgccactt	gctaagtaac	-36
		ttgttttggg	aatttggttt	cagATCACAT	TAACATGGT	GAAGGCTGTT	TGTGTGCTTG	25
		GTAGCAGTGA	GATGTTAAG	GGCACTATCC	ACTTGTGCTA	AGAGGGAGAT	Ggtatgccat	85

B	BanHI	GGATCTGAC	TTTCAGCAAT	TTGGTTAGGA	TTTCTGATGA	TGAAACACAG	ATTAATGATA	-2069
		ACTTGAAGGT	TGGTATTGTA	TTGTCCTCT	TGAGCTTATT	CATTGTAGAT	ACCGGTAAAT	-2009
				PstI				
		CCTCATATTA	CTTCTGCAGA	TTGTTGTACA	CCTTCCAAAC	AAACAACAAA	CTGTAGGGAC	-1949
		ATTGCAACAT	TATCATCTGC	ATTACAATGT	TGCTATTGTC	AGCATCAAGG	GTTCCTCGTG	-1889
		TCTTCGTACG	GAAGAATTTC	ATGATCCAGG	GCAAAITTAAG	TGTAAGAAGG	TATTATCTGT	-1829
		GGGACGTGCT	TTCAGCTCAG	GCAAAITTAAT	GGCCACAGGT	GGGATATTGA	CGGCAAAACC	-1769
		AAGCAATGCT	CACTGCAAGG	AACITATGCT	CTCCACTTGT	AGATCACTGA	AGTACACTGT	-1709
		AACTCCCAAA	TTGAAGTCTT	GCTTTTCTCT	GCAAAAGAAA	GGCTTTAATT	TTCTTATATT	-1649
		GGAAATAATT	TGTTGCTCTA	CAATATGAAC	AAGAGCAATT	TAATATAACA	CTACCAAAAC	-1589
				Apal				
		TTGCCATGTC	TGCTTACCAA	ACCTAGCCAA	AGGGCCCGCT	TAATAAATTT	ATGGCCATAC	-1529
		CTTAGGCAAG	ATTITGGGAAA	TAAATGAGTG	CATGACATAT	GTTCCTTAGG	ATTAAAGAAA	-1469
		CGGAATGAA	ACAACCTGTT	TACGAAGTGT	GGAAAGATTG	GCAAAAGTGT	AAACGCTCCT	-1409
				Apal				
		GCTTCTGCCG	AACATCTGCT	GTAATCCTTT	GTITTTCTGC	TAGGCTGGGA	TTGAGGGGCC	-1349
		CTTGATTTGAT	TTTGATGGGA	ATTTCGTTGG	TATGTAGGTA	AGGAGACTCT	GTATTATCCA	-1289
		AGGGATACAA	TTTCTGAATT	ATTGAGCTAT	TTTGATGGAG	AAGGGTATGT	CTTCTATGTT	-1229
		TATATGTATA	CTGTGTTTTC	CAACTTTTTC	ATGCTCTGTT	ATTGATGAGG	AGTAACAAAT	-1169
		TCACATTTTC	GTGCTCTTGA	TCGAGGGAAT	TCCTCTTGA	GATCACTGAG	AGTAACAAAT	-1109
		CGTCCCTCTG	TCGCAAGATA	CGTTGGCATT	ATCTCTGAGT	CTCAAGCACA	CGCACTGAGC	-1049
		AGAAGTGTGG	TTAATATGAA	AAGTACTAGA	TGAGACCTCT	CGCTTTGCA	CGCGGAATCT	-989
		TTCTGTATTA	TATTAATAAT	TCTAAATTGA	TATGCTGATA	CATATTAGTA	AAATATTGAA	-929
		ACTGATTAA	AAAGGAACCT	GATGCTATCT	ATACAAAAGA	CTCACTAAT	GAACGAAGA	-869
		GTCACTGCTC	CTAAGACATT	TTGAATGGGC	CTAGCCATCA	GATTTTGTAT	TAACAGACAG	-809
				EcoRV				
		GGAGTCTTAT	CGGAGAGGGA	GTAGGTAACC	GAGATATCTT	CTGATGAGAA	TAAGCAAAAT	-749
		CTATTATTTT	TTGTTGAAGA	AAAGAAAAGC	GTGTCACAGT	GAAGTGGACT	CTGAAATTTT	-689
				EcoRI				
		CCTCTCTCTC	TCTGAGATCT	TCTCATCAG	AAATCAGAAG	AGGAGAGGGT	GGGCAACTCG	-629
		CAGATCGCCT	TCTCGTCGCG	CTCGCGCCGC	AGGGGTCGCG	TGAAGtatgc	agcttcaact	-569
		cccccaactt	tctaggggttc	taactgcctc	tgtctgcctc	gattatgcgt	ggtgggtatg	-509
		cgctctcgct	cgccggagta	gctggatctg	tgtgccccgt	gtaattttag	gttttgggtt	-449
		cgatctggcc	gcgctctcgt	ttgttctcgc	cgctgtatgc	ttcgttccgc	catagggctc	-389
		atctcaggct	gcgcatctgg	acggccaaca	acataaaaaa	tccttgttaa	attatcggtt	-329
		catgtggtag	gggggctctt	caggatcgga	ggtttaggtg	atttggagta	gcaaacacgat	-269
				EcoRI				
		tttgcgtgtg	tagagagattc	gagaactctg	gcgcgcagat	gctaatttag	tggtttttaa	-209
		caaatgttaa	gacatcctga	tattatttgc	tgccgttaaa	gttcaaaact	tttgcaacggg	-149
		tgttctctag	ctataaagga	tatgagcata	tagtaccagg	tcttatcggt	ttgttctcat	-89
		acatacaatt	tgtctgattt	tatggacca	tcatactgag	atatataac	ctgtgcacct	-29
				NheI				
		ttcggtctct	gtgcagAAC	CATAGACAAT	GGTGAAGGCT	GTTGCTGTGC	TTGCTAGGAG	32

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 (GAANNNTTC) are underlined. Direct repeat sequences are shown in italic letters. A complementary sequence to the mutated oligonucleotide primer used for p*SodCc1*-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.

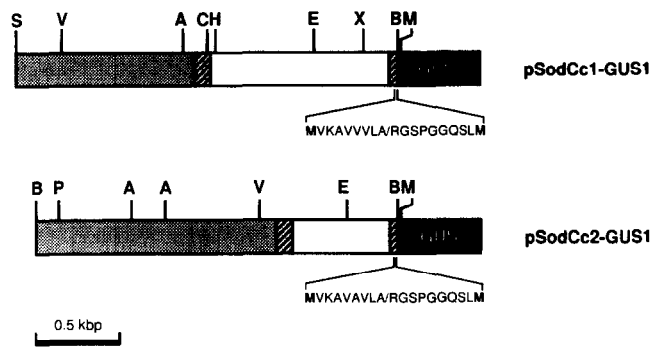


Fig. 3. Two *SodCc*-GUS chimeric constructs (pSodCc1-GUS1 and pSodCc2-GUS1) used for transient transformation of rice protoplasts. Hatched and open boxes refer to exons and introns, respectively, while shaded boxes indicate the 5'-flanking regions. The GUS coding sequence shown by a closed box is not 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, *Hind*III; 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 (GA₃) often function as antagonistic regulators, we examined whether the effect of ABA in the *SodCc2*-GUS expression is hampered by GA₃. 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 μ M) were titrated to induce the full activity of the *SodCc2* promoter. Fig. 4B shows that 10 μ M ABA is sufficient to maximally induce the *SodCc2*-GUS expression. The expression of GUS activities was proportional to ABA concentration from 1 to 10 μ M. These results clearly demonstrated that hormonal regulation of the expres-

sion 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

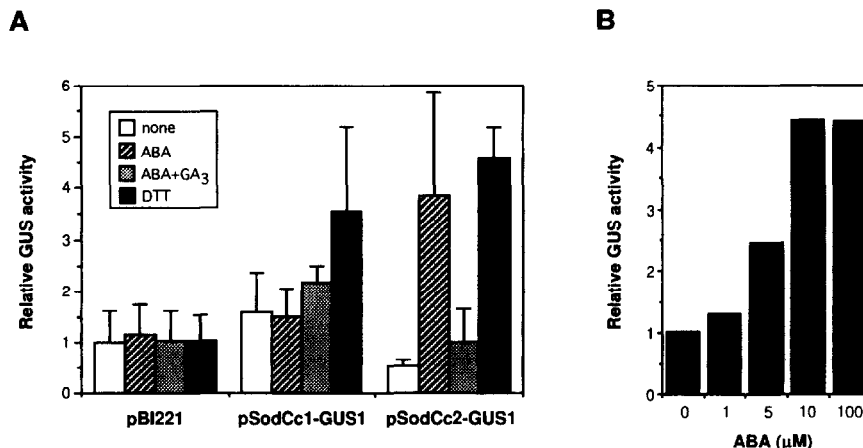


Fig. 4. Transient GUS expression in rice protoplasts transformed with chimeric constructs. (A) The effects of ABA and DTT on transient GUS expression from pBI221, pSodCc1-GUS1 and pSodCc2-GUS1. Transformed rice protoplasts were incubated at 28°C for 48 h without or with the following at the indicated concentrations: ABA, 100 μ M; GA₃, 100 μ M; 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 μ M 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 GA₃. 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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References

- [1] Bowler, C., Van Montagu, M. and Inzé, D. (1992) Annu. Rev. Plant Physiol. Plant Mol. Biol. 43, 83–116.
- [2] Asada, K., Kanematsu, S., Okada, S. and Hayakawa, T. (1980) in: Chemical and Biochemical Aspects of Superoxide and Superoxide Dismutase (Bannister, J. V. and Hill, H.A.O. Eds.) pp. 136–153, Elsevier, Amsterdam.
- [3] Hérouart, D., Bowler, C., Willekens, H., Van Camp, W., Slooten, L., Van Montagu, M. and Inzé, D. (1993) Phil. Trans. R. Soc. Lond. B 342, 235–240.
- [4] Perl-Treves, R. and Galun, E. (1991) Plant Mol. Biol. 17, 745–760.
- [5] Tsang, E.W.T., Bowler, C., Hérouart, D., Van Camp, W., Villarreal, R., Genetello, C., Van Montagu, M. and Inzé, D. (1991) Plant Cell 3, 783–792.
- [6] Sakamoto, A., Okumura, T., Ohsuga, H. and Tanaka, K. (1992) FEBS Lett. 301, 185–189.
- [7] Sakamoto, A., Okumura, K., Kaminaka, H. and Tanaka, K. (1995) Plant Physiol., in press.
- [8] Miao, Z. and Gaynor, J.J. (1993) Plant Mol. Biol. 23, 267–277.
- [9] Kardish, N., Magal, N., Aviv, D. and Galun, E. (1994) Plant Mol. Biol. 25, 887–897.
- [10] Hérouart, D., Van Montagu, M. and Inzé, D. (1993) Proc. Natl. Acad. Sci. USA 90, 3108–3112.
- [11] Hérouart, D., Van Montagu, M. and Inzé, D. (1994) Plant Physiol. 104, 873–880.
- [12] Sakamoto, A., Ohsuga, H. and Tanaka, K. (1992) Plant Mol. Biol. 19, 323–327.
- [13] Sakamoto, A., Nosaka, Y. and Tanaka, K. (1993) Plant Physiol. 103, 1147–1148.
- [14] Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) EMBO J. 6, 3901–3907.
- [15] Li, Z., Burrow, M.D. and Murai, N. (1991) Plant Mol. Biol. Rep. 8, 276–291.
- [16] Kosugi, S., Ohashi, Y., Nakajima, K. and Arai, Y. (1990) Plant Sci. 70, 133–140.
- [17] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
- [18] Marcotte Jr., Russell, W.R. and Quatrano, R.S. (1989) Plant Cell 1, 969–976.
- [19] Yamaguchi-Shinozaki, K., Mundy, J. and Chua, N.-H. (1990) Plant Mol. Biol. 14, 29–39.
- [20] Lam, E., Benfey, P.N., Gilmartin, P.M., Fang, R.X. and Chua, N.-H. (1989) Proc. Natl. Acad. Sci. USA 86, 7890–7894.
- [21] Gurley, W.B. and Key, J.L. (1991) Biochemistry 30, 1–12.
- [22] Skriver, K., Olsen, F.L., Rogers, J.C. and Mundy, J. (1991) Proc. Natl. Acad. Sci. USA 88, 7266–7270.
- [23] Bowler, C., Alliotte, T., De Loose, M., Van Montagu, M. and Inzé, D. (1989) EMBO J. 8, 31–38.
- [24] Kanematsu, S. and Asada, K. (1989) Plant Cell Physiol. 30, 381–391.
- [25] Williamson, J.D. and Scandalios, J.G. (1992) Proc. Natl. Acad. Sci. USA 89, 8842–8846.
- [26] Williams, M.E., Foster, R. and Chua, N.-H. (1992) Plant Cell 4, 485–496.