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Cloning and characterization of the rice *CatA* catalase gene, a homologue of the maize *Cat3* gene

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Abstract

We isolated and sequenced a genomic clone (*CatA*) encoding CAT-A catalase, a homologue of the maize catalase isozyme 3 (CAT-3) from rice (*Oryza sativa* L.). The 5′-upstream non-coding region had very low similarity with the maize *Cat 3* gene and possible *cis* elements and sequence motifs in the maize *Cat3* gene were not evident, except for TATA and CAAT motifs. Several sequence motifs found in the promoters of plant seed-specific genes were identified in the 5′-upstream non-coding region of the *CatA* gene. Northern blotting showed that the *CatA* gene is expressed at high levels in seeds during early development and also in young seedlings. Methyl viologen (paraquat) resulted in the 3-fold induction of the *CatA* gene in the leaves of young seedlings, whereas abscisic acid, wounding, salicylic acid, and hydrogen peroxide had no or only slight effects.

The 1.9 kb 5′-upstream fragment (−1559 to +342) of the *CatA* gene was fused with the *Escherichia coli* β-glucuronidase (GUS) gene and introduced by electroporation into protoplasts prepared from rice suspension-cultured cells, then the transient expression of the GUS gene was examined. Deletion analysis of this chimeric gene suggested that a weak silencer is located in the region between −1564 to −699. Abscisic acid (ABA) at a final concentration of 10^{-6} M doubled GUS activity in protoplasts electroporated with the chimeric DNAs having 1.9 to 1.2 kb 5′-upstream regions. A sequence highly similar to the Sph box, a motif found in genes modulated by ABA, was found at −266 to −254. Deletion of this region however, did not eliminate the responsiveness to ABA. Expression of the chimeric gene in the protoplasts was not enhanced by stress such as low and high temperature, hydrogen peroxide, methyl viologen, salicylic acid, elicitor, and UV light.

The chimeric CatA-GUS plasmid DNAs amplified in the methylation-positive strain, *E. coli* DH5α, showed GUS gene activities, whereas all the chimeric DNAs amplified in the methylation-deficient *E. coli* JM110 were completely inactive in the presence or absence of ABA in the culture medium. DNA methylation, especially of either one or both of the deoxyadenosines at the two GATC motifs (one in the first exon and the other in the first intron of the rice *CatA* gene), appeared to be responsible for the *CatA* promoter activity identified in the transient assay.

The nucleotide sequence data reported will appear in the DDBJ EMBL and GenBank Nucleotide Sequence Databases under the accession number D29966.

Introduction

Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) catalyzes the dismutation of hydrogen peroxide into oxygen and water, and it is found in a wide range of organisms from aerobic bacteria to higher plants and higher animals. Plant catalases are thought to play an important role, as an antioxidant defense gene, in response to environmental as well as physiological oxidative stress [44, 45]. So far, 17 cDNAs from 11 plant species have been cloned, and their sequences determined [53 and literature cited therein]. Genomic DNA clones encoding the maize CAT-1 [16] and CAT-3 catalases [1] and those of castor bean catalase [48] have been sequenced. Several additional partial sequences have been deposited in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases. Each of the catalase isozyme genes are expressed in a fairly complicated spatial and temporal manner throughout the life cycles of maize [6, 39, 44, 45] and of tobacco (*Nicotiana plumbaginifolia*) [53]. Microdissection of the maize *Cat1* promoter to find a nuclear protein-binding fragment has been recently reported [58].

We cloned a cDNA for a catalase isozyme (CAT-A) from immature rice (indica type) seeds 0–10 days after pollination [31]. The deduced amino acid sequence had overall similarity of about 72% to maize CAT-1, 59% to CAT-2, and 82% to CAT-3 catalase, respectively. Rice CAT-A therefore appears to be a homologue of maize CAT-3 catalase [31].

In view of recent findings indicating the roles of hydrogen peroxide in salicylic acid-mediated systemic acquired resistance in plants [10, 24], in elicitor-induced plant defense reactions [30], and also in response to low-temperature stress [36, 37], the mechanism of catalase gene expression in plant requires clarification. In this study, we isolated and characterized the *CatA* catalase gene from rice, analyzed its expression in various tissues, and investigated the putative promoter region by transient assay of the truncated promoter fused with a reporter gene in rice protoplasts.

Materials and methods

Plant materials and stress induction treatments

Rice seeds (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) were sterilized, soaked in water for 2 days at room temperature, sown in moist soil and grown in a greenhouse. To study the effects of ABA and various stress on the catalase mRNA accumulation in the leaves, leaves were excised from 15 2-week-old seedlings, floated in 100 ml of water, supplemented with various reagents where indicated in a Petri dish, briefly vacuum-infiltrated, and incubated in the dark for 8 h unless indicated otherwise. Reagent stock solutions were: 1 mM (±) 2-*cis*-4-*trans*-abscisic acid (ABA, a racemic mixture; Sigma, MO) in 50% (v/v) dimethylsulfoxide (DMSO), 1 mM methyl viologen (paraquat), 0.1 M salicylic acid (SA) in ethanol, and concentrated (about 30%) hydrogen peroxide (H_2O_2). Methyl viologen was applied in the dark and in the light (ca. 20000 lx). For wound induction, 2-week-old leaves were cut into about 1 cm long pieces and incubated for 8 h in water.

Genomic library and screening

A rice (*Oryza sativa* L., japonica type) genomic DNA library (Clontech, CA) in λEMBL3 was plated in the host *Escherichia coli* strain NM538 and phage plaques were transferred to nylon membranes (Hybond-N + : Amersham, UK). The DNA fragments to be used as probes to screen the library were prepared by amplifying the insert of a lambda recombinant phage which contains about 75% of the CAT-A catalase cDNA [31] by the polymerase chain reaction (PCR) [41], and ^{32}P-labeling using the Multiprime DNA labeling system (Amersham, UK) [14]. Hybridization with the radioactive probes proceeded according to standard protocols [42]. The membranes were washed in 2× SSC, 0.1% SDS at room temperature for 30 min, then in 1× SSC, 0.1% SDS at 68 °C for 1 h, air-dried, and exposed to X-ray film. After three rounds of screening, a phage plaque with a positive signal was purified. The

phage (λEM74/81) DNA was isolated from the bacterial lysate as described [42].

Subcloning, sequencing and sequence data analysis

The lambda EM74/81 DNA was digested with *Hin*dIII and *Eco*RI, and the 4.7 kb fragment that hybridized with the probe was subcloned into either pBluescript II KS + or SK + vectors (Stratagene, CA) digested with *Hin*dIII and *Eco*RI to obtain pBK74/81 and pBS74/81. Serial overlapping deletions were created with exonuclease III and mung bean nuclease [20] using an Exo III/Mung Deletion Kit (Stratagene, CA). The DNA was sequenced using a DNA sequencer model 373A (Applied Biosystems, CA) by dideoxy chain termination [43] using AmpliTaq DNA polymerase (Applied Biosystems, CA) and double-stranded plasmid DNAs. The sequence was determined for both strands of the inserts. Short nucleotide sequences were connected, translated into amino acids and compared with the maize catalase gene sequences using DNASIS (Hitachi, Tokyo) softwares. Nucleic acid sequence motifs were identified using DNASIS software from an in-house database constructed from published data [50 and literature cited therein].

Southern blots of rice genomic DNA

Total DNA was extracted from mature leaves of the cv. Nipponbare (japonica type) cultivar of rice as described [34] with slight modifications. A 10 μg portion of DNA was digested overnight with one of 18 restriction enzymes (see the legend to Fig. 2), resolved by electrophoresis in 0.8% agarose in TBE (89 mM tris(hydroxymethyl)aminomethane, 89 mM boric acid, 2 mM EDTA), blotted onto nylon membranes and hybridized with the ^{32}P-labeled probe described above for 16 h at 42 °C in 50% formamide, according to the standard procedures [42]. Membranes were washed twice in 2 × SSC, 0.1% SDS for 10 min at room temperature, once in 1 × SSC, 0.1% SDS for 15 min at 65 °C, and twice in 0.7 × SSC, 0.1% SDS for 15 min at 65 °C, then visualized by autoradiography at −80 °C.

Northern blots

To examine the rice *CatA* gene expression, total RNA was isolated from seeds sampled at 0–5, 6–10, 11–15, 16–20, 21–25 days after pollination (DAP), and also from 2-week-old seedlings, with or without various stress, using acid-guanidinium thiocyanate-phenol-chloroform as described [11]. Poly(A)$^+$ RNA was isolated from the seedlings as described [42]. Total RNA (20 μg) or poly(A)$^+$ RNA (0.5 μg) was resolved by electrophoresis on a 1% agarose gel containing formaldehyde, blotted onto a nylon membrane and hybridized with a ^{32}P-labeled probe at 65 °C in the hybridization mixture described above, but without formamide. Membranes were washed in 1 × SSC, 0.1% SDS for 20 min at room temperature, three times in 0.2 × SSC, 0.1% SDS for 20 min at 68 °C, then visualized by autoradiography at −80 °C. X-ray films were analyzed using an LKB 2222 UltroScan XL Laser Densitometer (Pharmacia).

Construction of CatA promoter-GUS reporter fusions

To transcriptionally fuse the *CatA* promoter and the GUS reporter gene, the pBK74/81 DNA was digested with *Bst*XI, blunt-ended, then with *Hin*dIII. The 1.6 kb 5′ fragment containing the putative promoter region (−1564 to +31, counting from the reported cDNA start site [31] as +1) of the rice *CatA* gene was inserted into the pBI201 (Clontech, CA) digested with *Hin*dIII and *Sma*I. A translational fusion was constructed by digesting pBK74/81 with *Eco*52I, blunt-ending and digestion with *Hin*dIII, then inserting the 1.9 kb 5′-upstream fragment (−1564 to +342) which contains the first exon, the first intron and a small fragment of the second exon, into the *Hin*dIII/*Sma*I digested pBI201. This plasmid was designated CatA-GUS-Δ0. Sequential 5′ deletions of

this plasmid were generated by consecutive digestion with *Hin*dIII and one of the four enzymes, *Bgl*II, *Ban*III, *Ppu*MI, or *Sfi*I. The DNA fragments were separated by agarose gel electrophoresis and the larger fragment containing a portion of the promoter, the GUS gene and the vector was recovered. After blunt-ending both ends, the DNA was self-ligated to obtain CatA-GUS-Δ1 (containing −699 to +342), CatA-GUS-Δ2 (−390 to +342), CatA-GUS-Δ3 (−174 to +342), or CatA-GUS-Δ4 (−148 to +342), respectively. These chimeric plasmids were used in transient expression studies. All plasmids were amplified in *E. coli* strain DH5α (*dam*$^+$, *dcm*$^+$), prepared by the clear-lysate procedure and banded once through CsCl gradients according to the standard method [42]. A portion of the plasmid DNAs prepared from strain DH5α was retransformed into strain JM110 (*dam*$^-$, *dcm*$^-$) to obtain non-methylated DNA.

Transient expression experiments

Suspension cultures of rice Oc cells [4] were maintained in 80 ml of amino acid medium [33], supplemented with 1 mg/l 2,4-D in a 300 ml flask, with gentle rotation (90 rpm) at 28 °C. Ten milliliters of the culture was transferred into 70 ml of fresh medium every week. Rice protoplasts were isolated from 5-day-old suspension cultured cells as described [26].

Aliquots (0.5 ml) of the protoplast suspension in EP buffer [46] were mixed with either chimeric plasmid DNA or pBI221 (both at 20 μg/ml) and carrier DNA (30 μg/ml salmon sperm DNA), then electroporated with a Gene-Pulser (BioRad, CA) at 300 V/cm with 500 μF capacitance and 100 Ω resistance in a 0.4 cm cuvette. After incubating for 10 min on ice, the protoplast suspension was mixed with 1 ml R2P medium [35], transferred to a Millicell (Millipore, CA) [26] in a 6 cm dish containing 5 ml of R2P medium, and cultured with or without nurse cells at 28 °C in the dark. After an incubation of 3 or 4 days, protoplasts were collected by centrifugation at 800 × *g* for 5 min, and stored at −80 °C. For the GUS assay, the sample was mixed with 200 μl of extraction buffer [22], and thawed in ice water for 5 min. The suspension was then sonicated for 1 min with a model 250 Sonifier (Branson, CT) with the output control set at 2 and a pulse frequency of 0.1 per second (10%). After centrifugation at 15000 × *g* at 4 °C for 5 min, the protein concentration [7] and GUS activity [22] were determined in the supernatant. Endogenous GUS activity was minimized by including 20% (v/v) methanol in the reaction mixture [25]. The results presented are average values from at least two experiments, each of which included triplicate samples.

Exposure of electroporated protoplasts to ABA, elicitor, and stress

Prior to exposure, electroporated protoplasts, containing CatA-GUS-Δ0 DNA, were incubated for 3 h at 28 °C in the dark for recovery. Protoplasts were exposed to various conditions in triplicate for the indicated periods at 28 °C in the dark, with the exception of the high- and low-temperature procedures, then collected by centrifugation, and used for the GUS assay. All the stock solutions of chemicals were filter-sterilized, and stored at −20 °C.

For ABA exposure, an appropriate volume of (±) 2-*cis*-4-*trans*-abscisic acid (a racemic mixture), was added to cultures from either a 10^{-3} M or 10^{-4} M stock solution in 50% (v/v) DMSO.

Other chemicals were diluted and added as follows: an appropriate volume of N-acetylchitoheptaose (an elicitor) [60] from a 1 mg/ml stock solution in water; a 10^{-3} M stock solution of methyl viologen (paraquat) in water; 10^{-2} M hydrogen peroxide diluted from a fresh 2×10^{-1} M stock solution, and 10^{-2} M salicylic acid in ethanol prepared from a 1 M stock solution in ethanol.

For exposure to UV light, the cultures were irradiated, without plastic covers, at a distance of 25 cm from a UV lamp (sterilization lamp GL-15; Hitachi, Tokyo) on a clean bench for 5 min. The protoplasts were then incubated at 28 °C in the dark for 3 days. For exposure to high and low

temperature, the cultures were incubated in the dark either at 16 °C or 42 °C for 1 day, then incubated at 28 °C for 2 days in the dark.

Results

Nucleotide sequence of a rice CatA catalase genomic clone

The probe used to screen the genomic library was the 1.4 kb PCR product of lambda clone 51, which contains a cDNA encoding the major portion of rice CAT-A catalase [31] flanked by a 3′-noncoding sequence. After the third screening, we obtained six positive lambda clones that gave signals of almost equal intensity. The insert DNAs were then excised and subcloned into pBluescript vectors. Partial sequencing revealed that they were almost identical to the cDNA, and therefore these genomic clones encoded CAT-A catalase.

The complete nucleotide sequence of 4676 bp from clone pBK74/81 and the derived amino acid sequence are shown in Fig. 1. Four exons and three introns were identified by aligning the genomic sequence with that of the *CatA* cDNA [31]. All three exon-intron junctions had the usual AG-GT motifs and the intron-exon junctions were either AG-TT, AG-GT or AG-TG. There were 4 discrepancies in the nucleotide sequences in the coding regions: nucleotides +657, +679, +1747, and +1750 in the genomic sequence were all Cs, but the corresponding nucleotides in the cDNA are A, G, G, G, respectively [31]. All except for the second difference, would result in amino acid changes (Gln-113, Ala-448, and Val-449 in the cDNA [31], and Pro-113, Pro-448, and Leu-449 in the genomic sequence), while the other would yield the same amino acid residue. Whereas most plant catalases to far characterized have Pro at residue 113 (numbering in [31]), the maize CAT-3 [39] and rice CAT-A [31] cDNAs had Gly and Gln, respectively. It is not clear whether the reported cDNA sequence of rice CAT-A is incorrect, or whether this position is variable in CAT-3 type isozymes in monocots. Two other amino acid residues were also located in the variable region, and therefore it is not clear whether these discrepancies were due to technical errors or a difference between japonica and indica subspecies.

Genomic Southern blots

An autoradiogram of the Southern blots of rice total DNA digested with one of 18 restriction enzymes showed one or two major bands and in some samples, an additional minor band, suggesting one to a few copies of the *CatA* gene and a related sequence with relatively low similarity in the rice genome (Fig. 2). The minor band in the *Bam*HI digests showed a restriction fragment length polymorphism between indica-type and japonica-type cultivars [23]. Thus, by linkage analysis of the minor band segregation in *Bam*HI-digested genomic DNA from 144 individual F2 plants resulting from a cross between Kasalath and FL134, we identified and mapped one locus for a catalase gene (*Cat-A1*) in rice [23]. This was not the locus of the *CatA* gene reported here, but rather that for one of the small gene family of rice catalases, having some similarity to the *CatA* gene sequence.

Expression of the rice CatA gene

A transcript of about 1.9 kb was detected in all the tissues examined (Fig. 3A). The relative abundance of the transcript was higher in seeds at the very early stage (seeds sampled at 0–5 and 6–10 DAP) than those at the middle (11–15 and 16–20 DAP), or late stages (21–25 DAP). Although the amount in the young seedlings could not be compared with those in the seeds because poly(A)$^+$ RNA was used instead of total RNA, it seems likely that rapidly dividing cells such as those in rice embryos and endosperm at the early developmental stage express high levels of the *CatA* gene.

We examined the effects of ABA and various stress on the expression of *CatA* gene in the young leaves (Fig. 3B). ABA at a final concentration of

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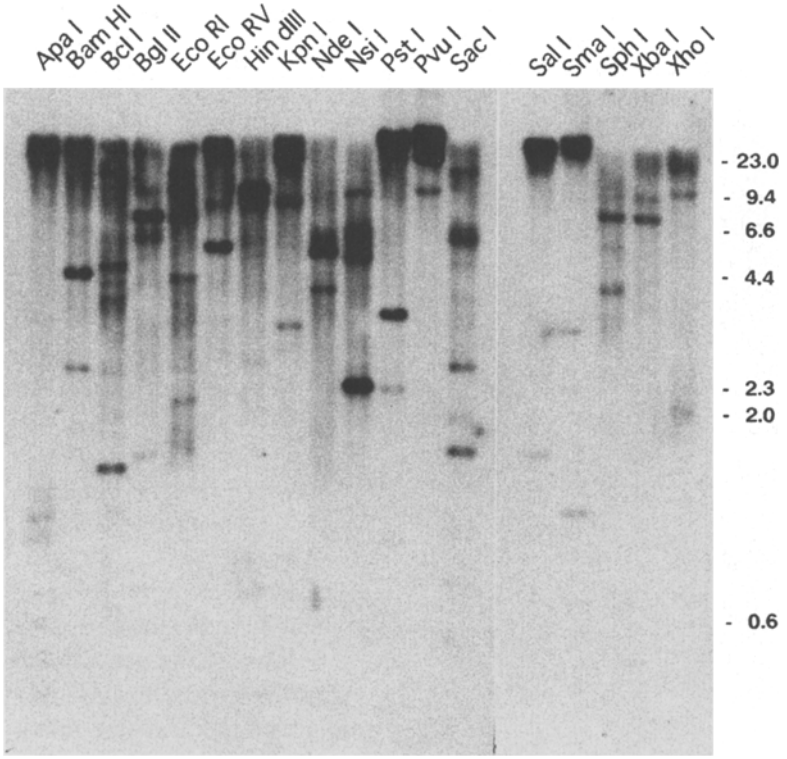

Fig. 2. Southern blots of rice genomic DNA. Rice total DNAs were digested with one of 18 restriction enzymes (*Apa*I, *Bam*HI, *Bcl*I, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Nde*I, *Nsi*I, *Pst*I, *Pvu*I, *Sac*I, *Sal*I, *Sma*I, *Sph*I, *Xba*I, or *Xho*I), resolved by electrophoresis, blotted onto nylon membranes, and hybridized with the ^{32}P-labeled probe derived from a partial *CatA* cDNA.

10^{-5} M (ABA), methyl viologen (paraquat) at 10^{-5} M in the dark (MVD), wounding (WD), salicylic acid at 10^{-4} M (SA), or hydrogen peroxide at 10^{-3} M (HP), either had no discernible effect or slightly enhanced the accumulation of *CatA* gene transcripts compared with the control (CD). Densitometry showed that the bands intensities were in the range of 0.8 to 1.3 relative to the controls. The levels in the leaves exposed to water in the light (CL) was also in this range (data not shown). Methyl viologen at a final concentration of 10^{-5} M in the light resulted in a higher level (about 3-fold by the densitometric analysis) of *CatA* gene transcripts (Fig. 3B, lane MVL).

Because the probe contained coding as well as non-coding regions, the possibility cannot be excluded that the detected bands represent those of other catalase genes, although the conditions for

Fig. 1. Nucleotide and derived amino acid sequences of the rice catalase *CatA* gene. The 5′ end of the CAT-A cDNA [31] is indicated as + 1. Upper case denotes nucleotides in the mRNA; the predicted amino acids in three-letter code are shown below the respective codons. Lower case denotes the sequence 5′ of the putative transcription start site (+ 1), introns, and that 3′ of the putative transcription termination site. Potential polyadenylation signals are underlined. In the 5′ non-transcribed region, consensus TATA (−72 to −66) and CCAAT (−257 to −253) boxes, and a CATGCAT box (−264 to −258), an Sph box-like sequence (−266 to −254), a lectin box (−659 to −653), and a glutenin box (−881 to −874) are underlined. Furthermore, a highly conserved region (−231 to −197) between the genomic sequences of rice *CatA* and maize *Cat3* [1], a 12 bp direct repeat (−1441 to −1430 and −550 to −539), a 10 bp indirect repeat (−1382 to −1373 and −21 to −12), an 11 bp indirect repeat (−826 to −816 and −63 to −53), a 10 bp inverted repeat (−1440 to −1431 and −549 to −5401), an 11 bp inverted repeat (−1276 to −1266 and −816 to −806), a 12 bp inverted repeat (−1269 to −1258 and −1204 to −1193), and a 10 bp inverted repeat (−1068 to −1059 and −712 to −703) are also underlined (see Discussion).

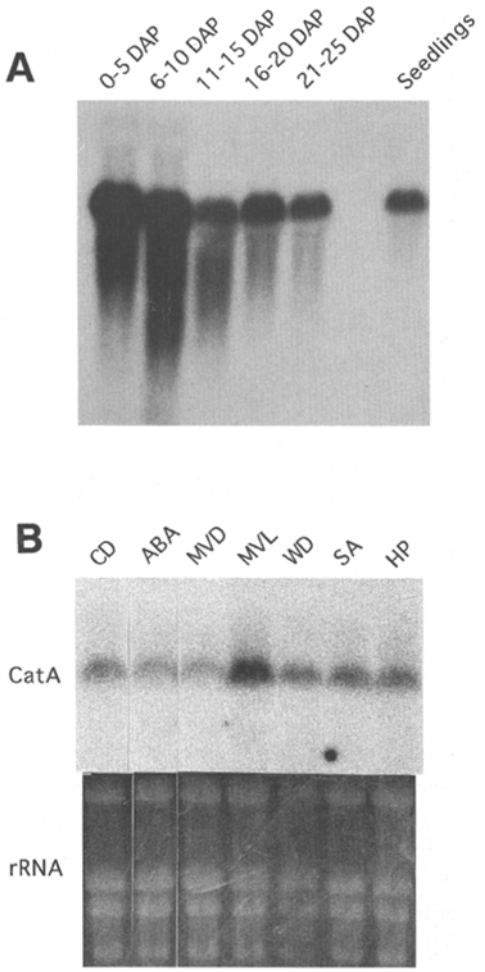

Fig. 3. A. Northern blots of *CatA* RNA accumulation in various rice tissues. Total RNAs prepared from the seeds at various days after pollination (DAP) (0–5, 6–10, 11–15, 16–20, and 21–25 DAP), and poly(A)$^+$ RNA prepared from two-week-old seedlings were analyzed. B. The effects of ABA and stress on the accumulation levels of *CatA* gene transcripts in leaves (upper panel). Young leaves were exposed to water (control; CD), 10^{-5} M ABA (ABA), 10^{-5} M methyl viologen (MVD), 10^{-5} M methyl viologen in the light (MVL), wounding stress by cutting into small pieces (WD), 10^{-4} M salicylic acid (SA), or 10^{-3} M hydrogen peroxide (HP). All exposures proceeded in the dark, unless otherwise indicated, for 8 h. The loading of equivalent amounts of RNA from leaves was verified by staining the gels with ethidium bromide (lower panel).

hybridization and washing the membrane were rather stringent. However, since our preliminary results showed that a cDNA for rice CAT-B catalase [32, 52] and the 3′ noncoding region of the rice *CatC* genomic clone [unpublished data] hybridized to genomic DNA fragments that differed from those that hybridized with CAT-A cDNA (data not shown), it is likely that the transcripts of these three genes were distinguished under our experimental conditions, and that the signals detected in Fig. 3 represented transcripts of the *CatA* gene. Preliminary northern blots using the presumably *CatA* gene specific probe (3′ non-coding region, 1531–1835, of CatA cDNA [31]) showed a faint but identical profile to that shown in Fig. 3B (data not shown).

Transient expression analysis of promoter deletion constructs in rice protoplasts

The putative promoter region (−1564 to +342) which includes the first exon, the first intron and a portion of the second exon of the *CatA* gene, was fused with the GUS gene in plasmid pBI201 to form the translationally fused gene, CatA-GUS-Δ0. This was introduced into protoplasts by electroporation, and we examined its transient expression. GUS activity begin to appear after a 2-day incubation, and the maximum enzyme activity was found 3–4 days after electroporation. The promoter activity of the *CatA* gene was almost double that of cauliflower virus (CaMV) 35S RNA promoter of pBI221 in the protoplasts prepared from rice suspension-cultured cells (Fig. 4).

We produced sequential deletions from the 5′ end of this fusion and examined their promoter activities. CatA-GUS-Δ1 (GUS gene fused with the region −699 to +342 of the *CatA* gene) was more active than the promoter in Δ0, therefore there may be a weak silencer in the region between −1564 to −699. The promoter activities gradually declined as the 5′-upstream region became shorter in Δ2 (−390 to +342), Δ3 (−174 to +342), and Δ4 (−148 to +342).

The effects of ABA on transient expression

Adding 10^{-6} M ABA to the medium during the post-electroporation culture of the protoplasts

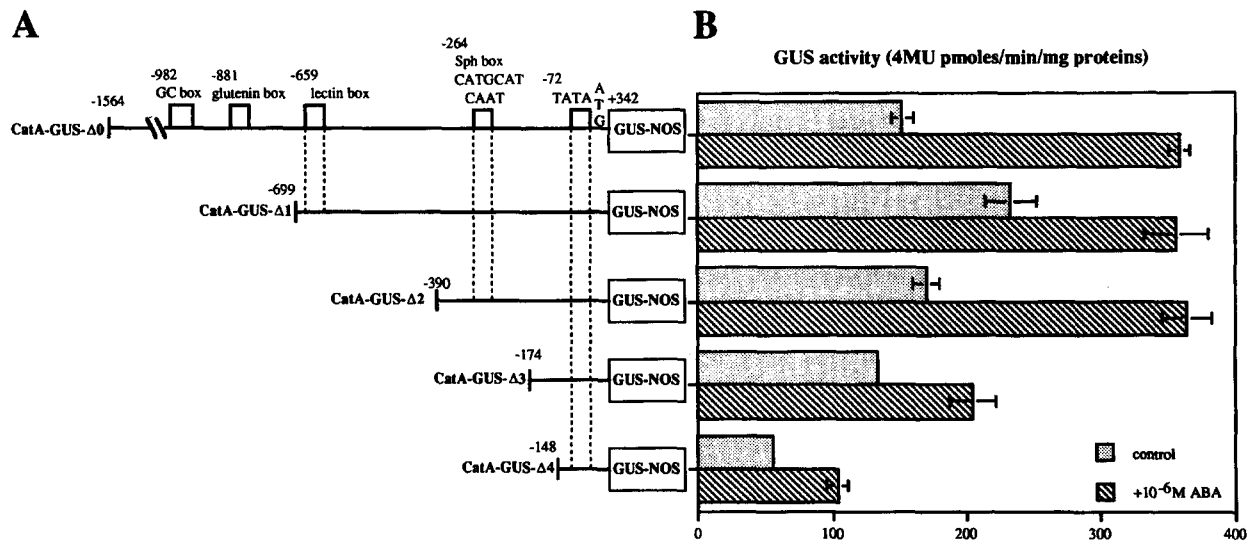

Fig. 4. A. Structures of the CatA-GUS-Δ0 construct and a 5′ deletion series (from Δ1 to Δ4). B. The effects of progressive 5′ deletions of the catalase *CatA* promoter on transient GUS gene expression in transformed rice protoplasts in the presence or absence of 10^{-6} M ABA. GUS was assayed after 4 days, and is shown in pmol 4-methylumbelliferone (4MU) per minute per mg protein. The horizontal bars give the standard errors of the means ($n = 3$). The GUS activity with pBI221 DNA was about 85 pmol min per mg protein in this series of experiments. Cultures with or without nurse cells gave similar results in all the samples.

containing CatA-GUS-Δ0 DNA doubled in GUS activity after 4 days (Fig. 4). This was similar to the increased accumulation (2- to 3-fold) of transcripts of the manganese-superoxide dismutase (Mn SOD) genes in maize embryos [62] and the 7S globulin gene transcripts (5-fold) in wheat embryos [56]. These results suggested that rice *CatA* gene expression is modulated by ABA *in vivo*.

ABA at a concentration of 10^{-5} or 10^{-4} M increased GUS activity only slightly over 10^{-6} M (data not shown). Because an Sph box-like sequence was identified at −266 to −254 by computer analyses (see Discussion), we compared the responsiveness to ABA of the short constructs, *i.e.*, CatA-GUS-Δ3 (−174 to +342) and CatA-GUS-Δ4 (−148 to +342) DNAs, in which the box was deleted, with that of the longer constructs (Fig. 4). While the overall GUS activity of the deleted clones decreased, the difference in control and ABA-treated protoplasts was still about 2-fold in the short constructs.

However, the possibility that the ABA induction found here is a secondary effect caused by differences in the growth of protoplasts in the presence of ABA, rather than induction at the transcription level cannot be excluded, although rice protoplasts with nurse cells reportedly start dividing after 5–6 days, and nurse cells are absolutely required to induce this division [26].

The effects of exposing protoplasts to various stress on transient expression of CatA-GUS-Δ0 DNA

The exposure of electroporated protoplasts to various types of stress generally reduced the GUS activity (Fig. 5). The following were all performed in the dark and did not have large detrimental effects on the expression of the GUS gene: (a) 16 °C for 1 day, then 28 °C for 2 days; (b) 10^{-6}, 10^{-5}, or 10^{-4} M hydrogen peroxide (H_2O_2) for 4 days at 28 °C; (c) 2×10^{-7} or 2×10^{-6} M methyl viologen (paraquat) for 3 days at 28 °C; (d) 10^{-5} or 10^{-4} M salicylic acid, 4 days. However, the possibility that the damage inflicted by these stressors was recovered at 28 °C cannot be excluded.

On the other hand, the following reduced the GUS activity by more than about 50%: (a) 42 °C for 1 day, then 28 °C for 2 days; (b) 0.1 mg/ml or

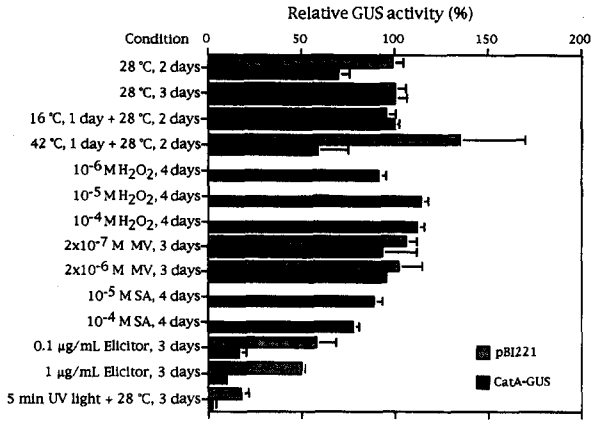

Fig. 5. The effects of exposing electroporated protoplasts to stress on the transient expression of CatA-GUS-Δ0 DNA. GUS activities are shown relative to those of the extracts prepared from the control protoplasts incubated for 3 days at 28 °C after electroporation. The enzyme activity using CatA-GUS-Δ0 DNA, was maximal 3–4 days after electroporation. For exposure to high or low temperatures, the protoplast cultures were incubated in the dark either at 16 °C or 42 °C for 1 day, then incubated at 28 °C for 2 days in the dark. For the chemical treatments, the cultures were exposed to the following for the indicated periods at 28 °C in the dark: H_2O_2, hydrogen peroxide; MV, methyl viologen (paraquat); SA, salicylic acid, Elicitor: N-acetylchitoheptaose. UV light: the cultures were irradiated at a distance of 25 cm from a UV lamp (sterilization lamp GL-15) on a clean bench for 5 min, then incubated at 28 °C in the dark for 3 days. The protoplasts containing pBI221 DNA were not exposed to H_2O_2 and SA.

1 mg/ml elicitor (N-acetylchitoheptaose) for 3 days at 28 °C; (c) UV light (25 cm) for 5 min, then 28 °C for 3 days. The reduced GUS activity could indicate not only down-regulation of the chimeric genes, but also the reduced viability of the protoplasts after exposure to the stress.

The exposure to 42 °C for 1 day increased the expression of the GUS gene under the control of CaMV 35S promoter, whereas those under the control of *CatA* promoter were suppressed to 60% of the control cultures. These data suggest that, contrary to the CaMV 35S promoter, that for *CatA* is not inducible by heat shock. Our studies with rice seedlings showed that rice heat-shock proteins are synthesized mainly during heat stress (42 °C for 2 h), and that the levels gradually decreased over 4 h at 28 °C [21].

The effect of methylation on the transient expression assay

The bacterial strain used for plasmid amplification influences the transient expression of plasmid DNA in plant protoplasts [51]. DNA methylation is thought to be partly responsible for deregulating promoter activity in transient expression systems [51].

We therefore compared the transient expression of the chimeric DNAs using *E. coli* strain DH5α (*dam*$^+$, *dcm*$^+$) and strain JM110 (*dam*$^-$, *dcm*$^-$) as hosts. The influence of the bacterial strain genotype was striking (Fig. 6). The pBI221 plasmid DNA, which contained the CaMV 35S promoter, reduced GUS activity by about 50% when the DNA was prepared from JM110 as compared with those from DH5α. On the other hand, the chimeric DNAs, from CatA-GUS-Δ0 to -Δ4, showed almost no activity (less than about 3% of those with DNA from DH5α). In other words, DNA methylation increased the *CatA* promoter activity 36-fold. ABA did not improve the gene activity of the CatA-GUS deletion series of DNAs amplified in JM110.

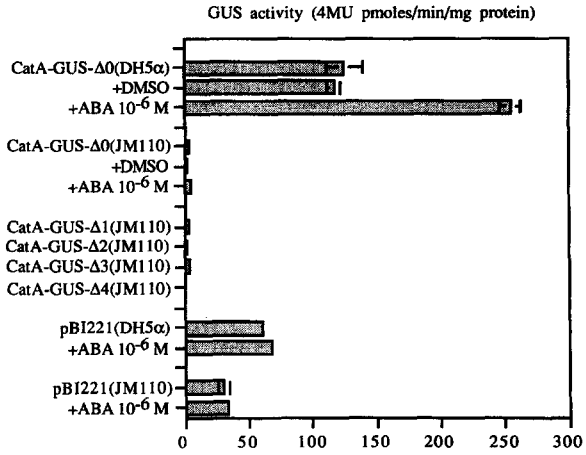

Fig. 6. The effects of plasmid DNA methylation on transient expression in rice protoplasts. Plasmid DNAs containing the chimeric CatA-GUS-Δ0 and the deletions (Δ1 or Δ4) or pBI221 were amplified in the bacterial strains DH5α (*dam*$^+$, *dcm*$^+$) or in JM110 (*dam*$^-$, *dcm*$^-$). After electroporation, the protoplast suspensions were incubated for 4 days in the dark at 28 °C, either in the presence or absence of 10^{-6} M ABA, then the protoplast extracts were assayed for GUS.

To examine the extent of DNA methylation, the CatA-GUS-Δ0 plasmid DNAs amplified either in *E. coli* DH5α or JM110 were digested with methylation-sensitive and insensitive restriction enzyme pairs, *Eco*RII-*Bst*NI for Dam (G*ATC; potentially methylated adenine indicated with *) and *Mbo*I-*Sau*3AI for Dcm methylation sites (C*C(A/T)GG). *Eco*RII and *Mbo*I cannot cut these sites when either the A or C is methylated, whereas the other enzymes cut regardless of the methylation status of these bases. DNAs were also digested by *Dpn*I, which cuts the Dam sites only when the adenine is methylated. These digests were analyzed by agarose gel electrophoresis (Fig. 7). The results confirmed that the cytosine and the adenine residues in the Dam or Dcm sites were highly methylated in the DNAs derived from *E. coli* DH5α.

Discussion

The rice gene encodes the counterpart of maize CAT-3 catalase

We isolated a rice genomic DNA fragment which codes for CAT-A catalase, a homologue of the maize gene coding for CAT-3 catalase, and determined the sequence of its 4676 nucleotides. In a dendrogram based on the similarity of either the deduced amino acid or nucleotide sequences of 17 plant catalase cDNAs so far published, rice CAT-A and maize CAT-3 catalases are unique and form an isolated branch ([53], K. Higo, unpublished). Potential dicot counterparts have not yet been reported.

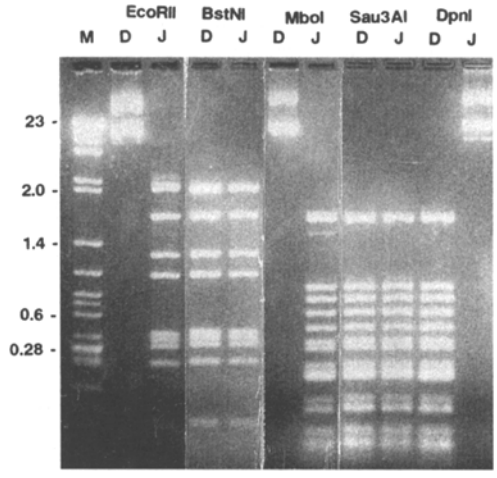

Fig. 7. Methylation analysis of the CatA-GUS-Δ0 DNA used for the transient assays. DNA was amplified either in *E. coli* DH5α (*dam*$^+$, *dcm*$^+$) or JM110 (*dam*$^-$, *dcm*$^-$) strains, purified as described in Materials and methods, and digested with the restriction enzymes as shown for 2 h. The digests were separated on a 1% agarose gel, and stained with ethidium bromide. For the enzyme specificity, see Results.

Maize CAT-3 catalase is unique in that it co-isolates with mitochondria [44, 45], not with peroxisomes or glyoxysomes as do most plant catalases. Maize CAT-3 catalase is found at high levels in the epicotyl and in green leaves, and at low levels in the mature scutellum and root [38]. CAT-3 and CAT-2 catalases have enhanced and low peroxidatic activities, respectively [19]. The levels of the *Cat3* gene transcripts vary diurnally in the root as well as in the leaves [2]. Although rice CAT-A catalase has not yet been characterized in detail, we presume a similar intracellular localization because CAT-3 and CAT-A have highly similar amino acid sequences. These types of catalase might be specific to monocots.

Structural features of the 5′-flanking region

Computer analyses of the sequence 5′ of the cDNA start site identified a putative consensus TATA box (TATATAA, −72 to −66) and a CCAAT box (CCAAT, −257 to −253) (Fig. 1). A CATGCAT box was found at the region −264 to −258. This sequence, also known as a 'legumin box', has been found in the promoters of most legume storage protein, and other seed-specific genes, and it is believed to modulate these expression [9, 27].

We also found a sequence that was quite similar to the Sph box [18] (TCCATGCATGCAC) in the region −266 to −254 (TCCATGCATCCAA, identical bases underlined). The region consisting of the Sph box and two proximal upstream GTGTC sequences may be involved in the ABA modulated expression of the C1 regulatory gene for anthocyanin synthesis in maize [18]. In the rice *CatA* gene, there were two proxi-

mal upstream TGATGA sequences between −283 and −270 (Fig. 1). We also found a lectin box (ATTAAAT, −659 to −653), which has been identified in the promoters of the lectin (another soybean storage protein) genes, and it is believed to modulate the expression during seed maturation [28]. In addition, we also found a glutenin box (TGAAAACT, −881 to −874) which was homologous to the cereal storage protein consensus sequence [12] and identical to a sequence found in the pea legumin gene [47]. All of these elements have been found in the promoter region of genes specifically expressed in seeds. Therefore, these sequences may be also involved in the expression of the rice *CatA* gene in seeds.

A computer search for repeated sequences revealed several direct, indirect and inverted repeats as described in the legend to Fig. 1. A duplicated 10 bp palindromic sequence (TAGCTAGCTA) at −549 to −540 and also at −1440 to −1431, overlapping a 12 bp direct repeat and a 10 bp inverted repeat (Fig. 1), may be noteworthy, but the biological significance is unclear.

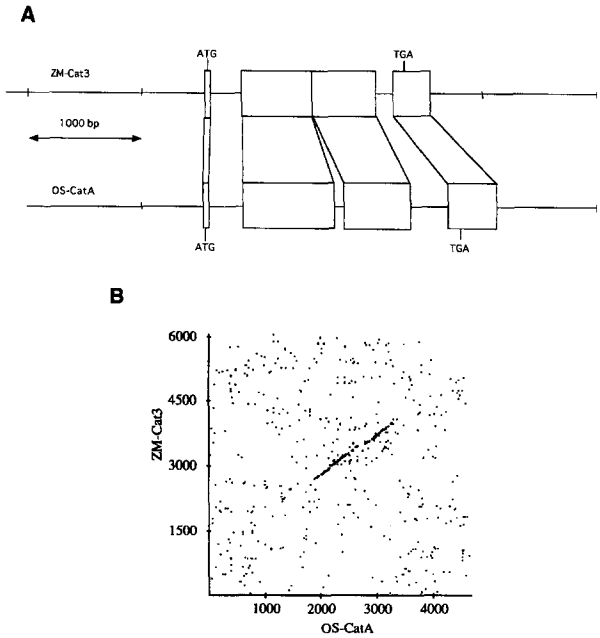

Fig. 8. A comparison of the structures of the rice *CatA* catalase gene (OS-CatA) and its maize counterpart, *Cat3* (ZM-Cat3) [1]. A. A comparison of exons and introns. Exons are indicated by white boxes. ATG: translation initiation site. TGA: translation stop codon. B. Dot matrix comparison of the nucleotide sequences of rice *CatA* and maize *Cat3* genes. An identity of 9 bases at a window size of 10 bases is shown as a dot.

Comparison with maize catalase genes

The rice *CatA* gene and its maize counterpart, the *Cat3* catalase gene [1], have 4 and 3 exons, respectively (Fig. 8). These exons are generally well conserved in length, but the maize counterpart of the second rice intron is missing. A comparison of the nucleotide sequences of both genes revealed no significantly long stretches of homology except for the coding regions, as shown in the dot plot (Fig. 8B). In the promoter region of the rice *CatA* gene, we found none of the several putative sequence elements reported for the maize *Cat3* gene promoter [1]. These included a direct repeat (AATCCCCTCCAATCCG/ATAT) around −200, three repeats of 7 bp (T/CCAATCC) around −206 to −180, a motif involved in the light-regulated expression of the monocot *cab* gene (AGCTCACC) at −121, and the ABA responsive element (ABRE; CACGTGGC), as well as the antioxidant responsive element (puGTGACNNNGC) around −2.4 kbp of the maize *Cat3* gene [1]. However, a careful inspection revealed that a stretch of 35 nucleotides is common in the two plant species. This sequence (TTTCCACACGAGGNGGGNCCNNNNCCACCTCANCT) is found at −231 to −197 in the rice *CatA* gene (Fig. 1) and at −341 to −306 of the maize *Cat3* gene [1]. Because this sequence was not found in the maize *Cat1* gene sequence [16] or the other two rice isozyme genes (*CatB* and *CatC*; unpublished data), it may be specific to the promoter of the gene encoding CAT-3 type catalase ('Cat3 element'). The function of this sequence remains unknown.

A comparison of the rice *CatA* gene sequence with that of maize *Cat1* gene revealed a common sequence similar to the GC box (GGGCTG, −982 to −977 in the rice sequence and −141 to −136 in the maize sequence [16]). This is believed to be the binding site for the general transcription factor Sp1 in mammalian cells [13].

However, these GC boxes in mammalian cells are within about 100 bp upstream of a TATA box. Therefore, the GC box-like sequence in maize and rice may not function as a binding site. No other conserved sequences were detected.

Effects of ABA and stress on CatA transcripts in the leaves of young rice seedlings

ABA at a final concentration of 10^{-5} M had no effects on *CatA* transcripts in young rice leaves (Fig. 3B). In the embryo, the maize *Cat3* catalase gene is not responsive to exogenous ABA [57], but in seedlings its expression is modulated by ABA [3, 37]. A sequence (CACGTGGGC) similar to that of ABRE is located about −2.4 kb 5′ upstream of the *Cat3* gene [1]. Although we did not find ABRE in the promoter region of the rice *CatA* gene, computer analysis revealed a Sph box-like sequence (see above).

Methyl viologen produces active oxygen species in the light. Whereas methyl viologen at 10^{-5} M in the dark had no effects (Fig. 3B, lane MVD), at the same concentration in the light, it induced *CatA* (Fig. 3B, lane MVL). Methyl viologen at a similar concentration induced one of three catalase genes in tobacco [54].

Salicylic acid was reported to bind catalase and inactivate it, resulting in the accumulation of active oxygen species, which are believed to be involved in the signaling for systemic acquired resistance ([10], and reviewed in [24]). Salicylic acid at 10^{-4} M had no effects on the level of the *CatA* transcripts (Fig. 3B, lane SA). Three tobacco catalase genes were also not induced in leaves by SA at similar concentrations (5×10^{-5} M) [54]. Salicylic acid at a concentration of 10^{-3} M resulted in the induction of three catalase genes in maize embryos [17].

Effects of the rice catalase intron on transient expression of the chimeric genes

In initial experiments, the putative promoter region (−1564 to +31) of the rice *CatA* gene was fused with the GUS gene in plasmid pBI201 to form a transcriptional fusion. This chimeric gene was introduced into protoplasts by electroporation, and the transient expression of the GUS gene was compared with that of pBI221 (the GUS gene under the control of the CaMV 35S promoter). Promoter activity of the *CatA* gene was almost undetectable in the protoplasts prepared from rice suspension-cultured cells (Oc cells), whereas the levels of activity were about 1/6 of that of CaMV 35S promoter in the protoplasts prepared from tobacco mesophyll cells (data not shown).

On the other hand, the translational fusion of the longer fragment (−1564 to +342) which includes the first exon, the first intron and a portion of a second exon of the *CatA* gene, to the GUS reporter gene resulted in almost double the promoter activity of the CaMV 35S promoter in protoplasts prepared from rice suspension-cultured cells. In other words, the activity increased about 12-fold by inserting the intron (Fig. 4). Increased foreign gene expression was first reported using the maize alcohol dehydrogenase (*Adh1*) intron in maize [8]. Similar results were later obtained in rice cells with the first introns of the rice actin [29] and triosephosphate isomerase [59] genes. Intron enhancement, however, appears to be limited to monocots [49, 59].

Northern hybridization of the RNA prepared from Oc suspension-cultured cells did not reveal rice *CatA* gene expression (data not shown). Therefore, the expression of the reporter gene under the control of the *CatA* promoter containing intron, may be due to the constitutive transcriptional activity of the *CatA* 5′-flanking region under the conditions of the transient assay. A similar relaxed regulation in the transient assay has been reported for the pathogenesis-related 1a protein gene [5] and the seed specific soybean gene [15].

Effects of ABA on transient expression of CatA-GUS chimeric gene

We examined the effects of ABA on the transient expression of the chimeric CatA-GUS genes in

rice protoplasts, and found that 10^{-6} M ABA almost doubled it (Fig. 4). Not much change was obtained by adding a higher concentration of ABA. It has been reported that the mRNA levels of several maize embryonic genes were also increased by exogenous 10^{-6} M ABA, but that culturing embryos with increasing concentrations of ABA little affected the mRNA levels [55]. The results of the transient assay using various deletion constructs (Fig. 4) showed that a deletion of the Sph box did not seem to eliminate the induction by ABA, suggesting that there is an element(s), other than ABRE or the Sph box, for responsiveness to ABA.

The putative promoter region of the rice catalase isozyme gene, *CatC*, responded differently to exogenous ABA in a transient assay, with respect to concentration dependence (unpublished data). This probably reflects the responsiveness of each catalase gene promoter to various concentrations of ABA in the cells, as has been reported for three maize catalase genes [57].

The effects of stress on the transient expression of the CatA-GUS chimeric gene

Most of the procedures applied here did not largely reduce or enhance the GUS activity (Fig. 5). The large reductions caused by the elicitor or UV do not necessarily mean the down-regulation of the CatA promoter-GUS chimeric gene, but it could be attributable to the reduced viability of the protoplasts exposed to these stressors. The differences in the GUS activity electroporated with pBI221 and CatA-GUS chimeric gene DNAs may be due to differences in the strength of the promoters. The reason we did not observe the increased accumulation of CatA-GUS transcripts may be that these protoplasts were already stressed so that further stress by active oxygen species for example, could not further induce the *CatA* gene. A similar concentration of the elicitor was sufficient for rice suspension cells to produce phytoalexins [60], but this concentration may damage to the rice protoplasts. UV irradiation of pea protoplasts for 5 min at a distance of 25 cm resulted in 2-fold induction of the phenylalanine ammonia-lyase gene promoter [61], but similar conditions appeared to be too harsh for the rice protoplasts.

The effects of methylation on transient expression

The influence of the bacterial genotype on the transient expression of CatA-GUS chimeric genes was powerful (Fig. 6). Similarly increased levels of the basal expression (and weak inducibilities) of several genes in plant protoplasts have been found with plasmids derived from a methylation-positive (*dam*$^+$, *dcm*$^+$) *E. coli* strain as compared with methylation-deficient (*dam*$^-$, *dcm*$^-$) strains [51]. The *dam* and *dcm* genes encode Dam and Dcm methylases, which methylate deoxyadenosine and deoxycytosine at specific sites, respectively. These are the only known DNA methylation systems acting on eukaryotic DNA in *E. coli*. A detailed transient assay in barley aleurone layers of *in vitro* methylated plasmid DNAs containing various promoters of differing strengths, indicated that Dcm methylation of the promoters had little or no discernible effect on the expression of the reporter gene, but that Dam methylation results in either about a 2-fold (CaMV 35S and maize ubiquitin promoters) or 15-fold (maize *Adh1* promoter) increase in the reporter gene expression [40].

While our preliminary northern analysis revealed no detectable expression of the *CatA* gene in rice suspension-cultured cells (data not shown), the CatA-GUS chimeric genes had about twice as much GUS activities than pBI221, when both DNAs were amplified in *E. coli* DH5α (*dam*$^+$, *dcm*$^+$). Methylation of the chimeric DNA caused about a 36-fold increase over that of the chimeric DNA amplified in *E. coli* JM110 (*dam*$^-$, *dcm*$^-$) in the protoplasts prepared from the same cultured cells (Fig. 6). This is the biggest increase caused by DNA methylation so far found in a transient expression assay.

Taken together, these results suggested that the CatA-GUS chimeric gene activity in the rice protoplasts found in this study is partly due to DNA

methylation during the amplification of the plasmids in *E. coli* DH5α. There is only one Dcm site (CC(A/T)GG) site at −252 and nine Dam (GATC) sites at −1130, −699, −420, −346, −338, −333, −328, +35 and +278 in the *CatA* promoter region of the chimeric constructs. Among these sites, the *CatA* promoter region in CatA-GUS-Δ4 (−148 to +342) contains only two Dam (+35 and +278) and no Dcm sites. Because the shortest construct CatA-GUS-Δ4 also showed an increased expression when the DNA was amplified in methylation-positive strain (Figs. 4, 6), increased levels of expression appear to be due to methylation of either one or both adenines in the GATC sequence at +35 (in the first exon) or +278 (in the first intron).

Although the possibility cannot be excluded that methylation acts via conformational changes of the DNA helix, as suggested [51], recent results favor the alteration of interactions of DNA and protein(s) involved in the regulation of gene expression, mainly mediated by methyldeoxyadenosine in the vicinity of the active promoter [40].

Acknowledgement

We thank Dr Naoto Shibuya, NIAR, for the kind gift of purified N-acetylchitoheptaose.

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