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Original Paper

New chimeric promoter useful for expression of selectable marker genes in rice transformation

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Abstract We developed a new chimeric promoter to express a selectable marker gene specifically at the selection stage during transformation of rice (*Oryza sativa* L.). The promoter consists of a 135-bp upstream sequence of the rice *RezA* gene and 4 or 8 copies of an enhancer fragment of the CaMV35S promoter. The β-glucuronidase (GUS) reporter gene under the control of this promoter was strongly expressed in transgenic rice calli but not in endosperm, the edible tissue in rice grains. When the hygromycin B phosphotransferase gene was fused with this chimeric promoter and introduced into rice, we were able to select transgenic plants. We demonstrated that the chimeric promoter did not influence expression of the endogenous *RezA* gene in the transgenic calli. These results indicate that this chimeric promoter could be useful for the selection of transgenic rice free of marker gene products in the edible tissue.

Key words: Callus, enhancer element, root, tissue-specific promoter.

In plant transgene technology, a selectable marker gene is usually co-delivered with the gene of interest to allow the efficient selection of transformants. However, the expression of selectable marker genes has raised public concern about the safety of transgenic plants, and the continued presence of marker proteins may compromise plant growth or productivity. To improve the agronomic performance of transgenic crops, especially food crops, several approaches to avoiding the accumulation of marker proteins in edible tissues have been reported (Ebinuma et al. 2001; Hare and Chua 2002; Permingeat et al. 2003). An alternative selection system using callus-specific promoters has been developed (Huang et al. 2001; Wakasa et al. 2003). Although this system is simpler than systems for removing the marker genes, only 2 kinds of such promoters have been developed so far. It is also desirable that the selectable marker gene be expressed in the root during the early stage of regeneration, in addition to the expression in the calli, to let root cells survive on selection medium.

In this report, we demonstrate that a new chimeric promoter consisting of the truncated *RezA* promoter and a tandem repeat of an enhancer-like element of the CaMV35S promoter is highly active in rice calli but not in seeds. We further demonstrate that the high activity of the new promoter had no effect on the expression of the endogenous *RezA* gene in callus cells, and thus allowed callus cells to develop normally. Our results show that the new promoter can be used to efficiently carry out selection in plant transformation.

Materials and methods

Construction of chimeric promoters

We retrieved the rice *RezA* gene genomic sequence (AP000570) from GenBank. We amplified a 2527-bp fragment of the 5′-upstream region (−2431 to +96, relative to the full-length cDNA starting site) of *RezA* by PCR from the genomic DNA of *O. sativa* cv. Nipponbare with primers P5 (5′-TCAAGCTTGACCGCT-ACCCTGGGCC-3′) and P6 (5′-GCTCTAGATCGG-ATCACTCGATCGGACGGC-3′). PCR was carried out in PCR SuperMix High Fidelity reaction mixture (Invitrogen Corp., Carlsbad, CA, USA). The cycle conditions were 30 s at 98°C, 30 s at 55°C, and 2 min at 72°C, for 25 cycles. The product was named RezA2527.

We produced a 5′-deletion series of the promoter by PCR from the RezA2527 fragment as follows. (1) A 997-bp (−901 to +96) fragment (RezA997) was amplified by PCR with primers P7 (5′-TCAAGCTTTTCT-AGGGAAGATAAAGCG-3′) and P6. The cycle conditions were 30 s at 98°C, 30 s at 55°C, and 1 min at 72°C, for 25 cycles. (2) A 478-bp (−382 to +96) fragment (RezA478) was amplified with P8 (5′-

Abbreviations: BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; GUS, β-glucuronidase; HPT, hygromycin B phosphotransferase; NAA, α-naphthaleneacetic acid; X-Gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid.

TCAAGCTTTAGGAACTAATTACACAACG-3′) and P6. The cycle conditions were 30 s at 98°C, 30 s at 55°C, and 30 s at 72°C, for 25 cycles. (3) A 233-bp (−137 to +96) fragment (RezA233) was amplified with P9 (5′-TCAAGCTTCCCTCGACCCGTCACG-3′) and P6. The cycle conditions were identical to those for the 478-bp fragment. (4) A 135-bp (−39 to +96) fragment (RezA135) was amplified with P10 (5′-TCAAGCTTCGCCTCTTTAAATGCG-3′) and P6. The cycle conditions were identical to those for the 478-bp fragment. Primers P5, P7, P8, P9, and P10 have *Hin*dIII sites, and P6 has an *Xba*I site. Each of the deletion sequences was thus obtained as a *Hin*dIII–*Xba*I fragment and was used to replace the CaMV35S promoter fragment of pCaMV35S-sGFP(S65T)-NOS3′ (Chiu et al. 1996) to produce pRezA2527-sGFP, pRezA997-sGFP, pRezA478-sGFP, pRezA233-sGFP, or pRezA135-sGFP, respectively.

The enhancer-like element (−351 to −84) of the CaMV35S promoter (Odell et al. 1988) was prepared by PCR from pBI221 with primers 35S-Sal (5′-CGTCGACAGGGCAATTGAGAC-3′) and 35S-Hind (5′-TCAAGCTTTGGAGATATCACATC-3′). The PCR cycle conditions were 30 s at 98°C, 30 s at 55°C, and 30 s at 72°C, for 25 cycles. The PCR product was obtained as a *Sal*I–*Hin*dIII fragment (hereafter referred to as "E"), ligated with RezA135, and inserted into pBluescript SKII (Stratagene, LaJolla, CA, USA) to yield p1xE-RezA135.

Tandem repeat construction was carried out essentially as described (Rushton et al. 2002). A 1xE-RezA135 fragment was excised from p1xE-RezA135 by *Sal*I and *Xba*I digestion and inserted into the *Hin*dIII–*Xba*I site of p1xE-RezA135 to yield p2xE-RezA135. By repeating this procedure, we obtained p4xE-RezA135 and p8xE-RezA135.

To examine the efficiency of selection with the chimeric promoter, we fused 4xE-RezA135 and 8xE-RezA135 each with the *HPT* gene. *HPT* was amplified by PCR from the binary vector pPZP2H-lac (Fuse et al. 2001) with primers HYGF (5′-CGCGGATCC-ATGAAAAAGCCTGAACTC-3′) and HYGR (5′-TAT-GAGCTCCTATTCCTTTGCCCTCGG-3′). The cycle conditions were 30 s at 98°C, 30 s at 55°C, and 1 min at 72°C, for 25 cycles. *HPT* was obtained as a *Bam*HI–*Sac*I fragment and used to replace the *GUS* gene in the binary Ti plasmid pBI121 (Clontech Laboratories, Inc., Mountain View, CA, USA) to obtain pBI-CaMV35S-HPT. To fuse the chimeric promoter with *HPT*, the CaMV35S was released from pBI-CaMV35S-HPT, and then 4xE-RezA135 or 8xE-RezA135 excised from p4xE-RezA135 or p8xE-RezA135 was inserted into the pBI-CaMV35S-HPT to obtain pBI-4xE-RezA135-HPT or pBI-8xE-RezA135-HPT.

To test the expression of the chimeric promoter histochemically, we fused 4xE-RezA135 and 8xE-RezA135 each with the *GUS* gene and inserted each fragment into pBI221, yielding p4xE-RezA135-GUS and p8xE-RezA135-GUS. These constructs were used for transient assay. To produce stable transformants, each construct was recloned into binary plasmid pPZP2H-lac to make pPZP-4xE-RezA135-GUS and pPZP-8xE-RezA135-GUS.

Transient expression analysis

Transient expression analysis was carried out with a helium-driven particle bombardment apparatus (Model GIE-III, Tanaka Co., Ltd., Sapporo, Japan). Physical factors for bombardment were set as described (Sugimura et al. 1999). Gold particles (0.03 mg; cat. no. 165-2263, Bio-Rad Laboratories, Inc., Hercules, CA, USA) with an average size of 1.0 μm were coated with 1 μg plasmid DNA and were bombarded into rice callus, root, and leaf with helium at 0.6 MPa. The bombarded tissues were incubated at 28 °C overnight in darkness on hormone-free agar medium that contained inorganic salts and vitamins (Murashige and Skoog basal medium with Gamborg's vitamins; Sigma-Aldrich, Co., St. Louis, MO, USA).

To evaluate the promoter activity of the successive 5′-deletion series, we looked for GFP fluorescence-activated cells through an IX70 fluorescence microscope equipped with an IX-FLA fluorescence attachment (Olympus Corp., Tokyo, Japan) with a GFP filter set (excitation wavelength: 460–490 nm; emission: 510–550 nm; dichroic: 505 nm), and used a 16-bit cooled CCD camera (Roper Scientific, Inc., Duluth, GA, USA) to capture the fluorescent cell images. After calibration of the digitized gray value against a gray-scale film, the cell images were traced on-screen and the fluorescence intensity was semi-quantified by using Scion Imaging software (Scion Corp., Frederic, MD, USA) and averaged. The values were the mean gray value of the pixels in the selected cell. The experiments were repeated three times under identical conditions. To see expression in the callus and leaf, we selected and measured at least 20 independent fluorescent cells for each successive 5′-deletion promoter and the CaMV35S promoter. To see expression in the root, we selected and measured 10 independent fluorescent cells bombarded with pCaMV35S-sGFP(S65T)-NOS3′, pRezA2527-sGFP, pRezA478-sGFP, or pRezA135-sGFP. The average fluorescence intensity was calculated to assess relative activity against that induced by the full-length promoter, pRezA2527-sGFP.

Histochemical GUS staining (Jefferson et al. 1987) after particle bombardment was done at 37 °C overnight in 50 mM phosphate buffer (pH 7.0) that contained 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc), 20% MeOH, and 0.1% Tween 20.

Stable transformation of rice

To examine the efficiency of selection with the chimeric promoter, we introduced pBI-4xE-RezA135-HPT and pBI-8xE-RezA135-HPT into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method (Holsters et al. 1978). Rice calli (*O. sativa* cv. Nipponbare) were transformed by *Agrobacterium*-mediated transformation as described (Hiei et al. 1994). Transgenic calli and regenerated plants were cultured on agar medium (Kyozuka and Shimamoto 1991) with 50 mg l^{-1} hygromycin B.

To see expression in callus, shoot, and root tissues, we introduced pPZP-4xE-RezA135-GUS and pPZP-8xE-RezA135-GUS into *A. tumefaciens* strain EHA101 by the freeze-thaw method as above. The culture conditions were identical to those for pBI-4xE-RezA135-HPT and pBI-8xE-RezA135-HPT.

PCR, RT-PCR and Southern analysis

We used PCR and Southern analysis to examine the integration of the fusion constructs in transgenic plants. Genomic DNA was prepared from leaves of regenerated rice, as described (Murry and Thompson 1980). PCR was performed in PCR SuperMix High Fidelity reaction mixture (Invitrogen Corp., Carlsbad, CA, USA). For Southern analysis, 10 μg of rice genomic DNA was digested with *Hin*dIII or *Eco*RI. Neither the *Hin*dIII nor the *Eco*RI restriction site occurs in either *GUS* or *HPT*. The *GUS* gene probe, which corresponds to the coding region, was amplified by PCR from pBI121 by using primers GUS-F (5′-GTCTGGTATCAGCGCGAAGTCT-3′) and GUS-R2 (5′-TCCATACCTGTTC-ACCGACGAC-3′). The cycle conditions were 30 s at 98°C, 30 s at 55°C, and 1 min at 72°C, for 25 cycles. The membrane was hybridized with the probe at 55°C, washed at 55°C, and visualized with the AlkPhos Direct Labeling and Detection System (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

For RT-PCR analysis, total RNAs from leaf, root, and callus were extracted with Sepasol (Nacalai Tesque Inc., Kyoto, Japan). DNase1-treated total RNA was reverse-transcribed with SuperScript III reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA) and oligo $d(T)_{15}$ (Gibco BRL, Gaithersburg, MD, USA). The first-strand products were amplified with a set of primers specific to *RezA* corresponding to 307 bp containing the 3′-UTR: RezRTF2 (5′-ACTCGGGTACGTGGTGAAACCG-3′) and RezRTR2 (5′-GACGACGACGCCATCCATCC-3′). For *GUS* mRNA detection, we used a set of primers corresponding to 499 bp containing the 3′-region: GUS-F2 (5′-ACTCAGCAAGCGCACTTACAGG-3′) and GUS-R2 (5′-TCCATACCTGTTCACCGACGAC-3′). The same samples were amplified with primers specific to the actin gene as a control. The RT-PCR cycle number was 25 in Figure 1 and 20 in Figure 5.

Analysis of DNA methylation

To examine the DNA methylation of the integrated *GUS* gene, we assayed levels of cytosine methylation by using the methylation-sensitive restriction endonuclease *Hpa*II and *Msp*I. Genomic DNA (10 μg) from transgenic rice was digested with *Hpa*II or *Msp*I and then hybridized with the labeled *GUS* gene probe.

Results and discussion

Construction of chimeric promoter capable of high expression in calli

Expression profile analysis during large-scale cDNA sequencing showed that the rice *RezA* gene is highly expressed in callus tissue (data not shown). The *RezA* cDNA sequence is registered in DDBJ/EMBL/GenBank (U46138), and is annotated as encoding a zinc-induced protein in rice without further details. A presumably full-length cDNA sequence with a longer 5′-UTR (AK069098) was isolated after it (Kikuchi et al. 2003). A BLAST search against the rice (cv. Nipponbare) sequence database (http://riceblast.dna.affrc.go.jp/) revealed that the copy number of *RezA* is 1, and the BAC clone containing the sequence is positioned at 43.2–45.4cM on chromosome 1.

To see whether this gene was expressed preferentially in callus, we compared the frequencies of the appearance of *RezA* transcripts among cDNA libraries prepared from callus, leaf, root, and germinating seeds at 24, 48, and 96 h after imbibition. *RezA* was highly expressed in callus but not in leaf or root (data not shown), as confirmed by RT-PCR analysis (Figure 1). Interestingly, *RezA* was expressed in the germinating seeds at 24 h after imbibition, and then expression sharply decreased at 48 and 96 h (data not shown), when metabolic activities accelerate (Takahashi 1995). The function of *RezA* has not been identified yet.

We retrieved the rice *RezA* genomic sequence (AP000570) from GenBank, and isolated RezA2527 (full-length promoter between −2431 and +96). To determine the region responsible for callus-preferential expression, we tested promoter activity by using 5′-deletion constructs (Figure 2A). RezA233 (truncated promoter from positions −137 to +96 relative to the cDNA starting site) and the longer promoters showed the same activity as RezA2527 in calli (Figure 3), but in leaves and roots they showed much lower activities. The promoter activity of RezA135 (−39 to +96) in calli was about half those of the longer promoters, and was nil in leaves and roots (Figure 3). These results indicate that callus-preferential *cis*-acting elements may exist within the 135-bp fragment. A computer search (PLACE database; Higo et al. 1999) of the

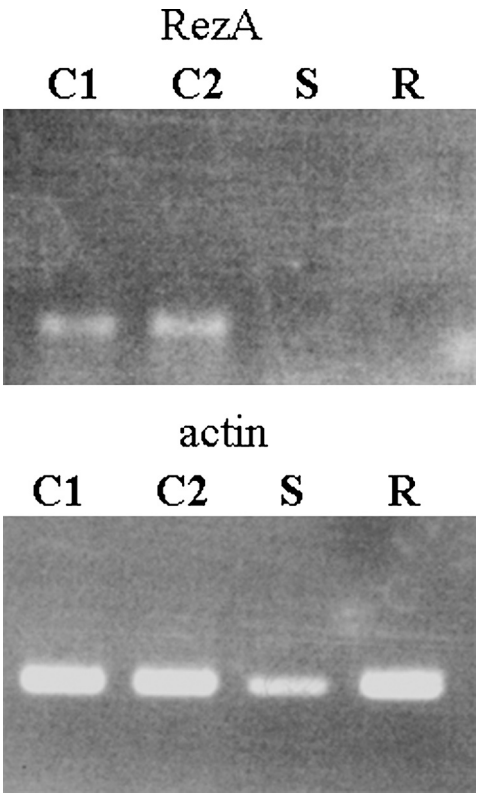

Figure 1. Tissue-specific expression of *RezA* gene in rice. Agarose gel electrophoresis was performed on RT-PCR products from calli incubated in regeneration medium containing 1 mg l^{-1} NAA and 2 mg l^{-1} BAP (C1) or 2 mg l^{-1} 2,4-D (C2), from shoot (S), or from root (R). The rice actin gene was used as a control.

5′-flanking region (−39 to +96) revealed no TATA-box-like motif. We found an initiator-like element (PyPyA(+1)N(T/A)PyPy: CCACAC) (Smale and Baltimore 1989) in the 5′-UTR region (+2 to +7) and a TCA motif near the initiator-like sequence (+13 to +73) (Nakamura et al. 2002). In some TATA-less promoters, initiator elements compensate for the lack of a TATA box and overlap with the transcription initiation site (Achard et al. 2003). Thus, RezA135 may be a TATA-box-independent promoter.

To strengthen the promoter activity of RezA135 for use in the selection of transgenic rice, we combined this fragment with multimers of the enhancer-like element from the 5′-upstream region (−351 to −84) of the CaMV35S gene (Figures 2A, B). In transient assay, fusion with 4 or 8 copies of the enhancer-like element increased the promoter activity (Figures 4A–C), as was reported for the CaMV35S core promoter (Mitsuhara et al. 1996). So far this is the first report to demonstrate a profound effect on a eukaryotic TATA-less promoter by a virus-derived enhancer.

Efficiency of selection and effect on endogenous gene expression of the chimeric promoter

To demonstrate the efficiency of selection, we introduced pBI-4xE-RezA135-HPT and pBI-8xE-RezA135-HPT into rice. Transformed rice calli were selected on agar medium containing 50 mg l^{-1} hygromycin B. Southern analysis indicated that all the regenerated plants carried the *HPT* gene (data not shown). We could not find any

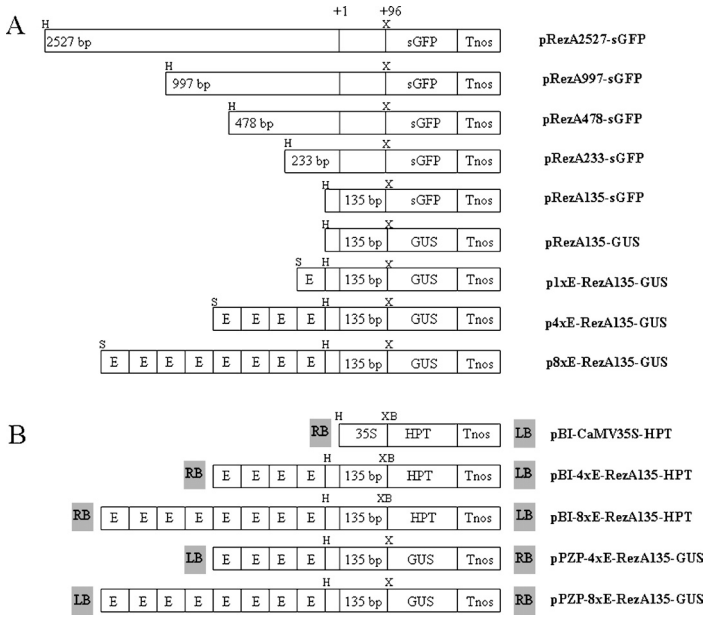

Figure 2. Diagrams of plasmid constructs used. (A) Constructs for transient expression analysis. All constructs were inserted into the polycloning sites of plasmid pUC18 or pBluescript SKII. (B) Constructs for transformation. Each construct was recloned into binary Ti plasmid pBI121 (pBI-CaMV35S-HPT, pBI-4xE-RezA135-HPT, pBI-8xE-RezA135-HPT) or pPZP2H-lac (pPZP-4xE-RezA135-GUS, pPZP-8xE-RezA135-GUS), as described in Materials and Methods. B, *Bam*HI; H, *Hin*dIII; S, *Sal*I; X, *Xba*I; RB, right border; LB, left border; E, enhancer-like element (−351 to −84) of the CaMV35S promoter; 35S, CaMV35S promoter; Tnos, polyadenylation signal of the gene for nopaline synthase in the Ti plasmid.

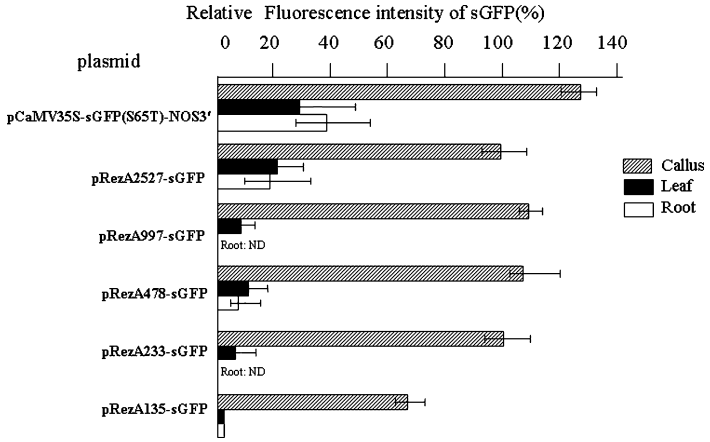

Figure 3. Transient expression assay of progressive deletion constructs of the *RezA* gene promoter. The constructs are depicted in Figure 2A. pCaMV35S-sGFP(S65T)-NOS3′ contains the *GFP* expression cassette, in which the cDNA of a modified *GFP* gene is under the control the CaMV35S promoter and the NOS terminator (Chiu et al. 1996). Error bars show standard error of mean. The experiments were repeated three times under identical conditions and gave essentially the same results.

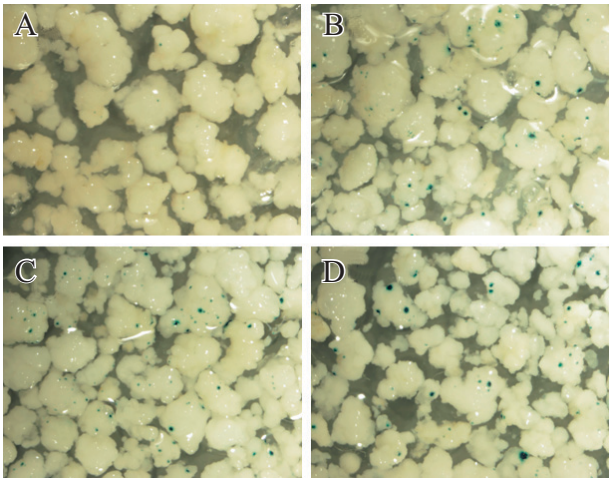

Figure 4. Transient expression of the chimeric promoter in rice calli. *GUS* gene expression in rice callus 1 day after particle bombardment. The plasmids contain pRezA135-GUS (A), p4×E-RezA135-GUS (B), p8×E-RezA135-GUS (C), and pBI221 (D). The experiments were repeated three times under identical conditions and gave essentially the same results.

differences between the efficiency of selection of pBI-4xE-RezA135-HPT and pBI-8xE-RezA135-HPT, although many more transformation experiments would be needed before we can draw a quantitative conclusion.

We then investigated the effect of the chimeric promoter on the expression of the endogenous *RezA* gene. As the chimeric promoter is specifically active in calli, we tested its effects on the expression of the endogenous *RezA* gene in the transformed calli. pPZP-4xE-RezA135-GUS and pPZP-8xE-RezA135-GUS were introduced into calli. As shown in Figure 5, the endogenous *RezA* was expressed at the same level in the non-transformant and in 6 transformants, in which the reporter *GUS* gene was expressed at various levels. Weak *GUS* signals were detected in lanes 1, 2, and 3. On the other hand, in the lane of the non-transformant, *GUS* expression was not detected. This result indicates that the chimeric promoter did not influence the expression of the endogenous *RezA* gene at all, and thus the chimeric promoter let callus grow and develop normally. Therefore, this promoter may be ideal for introducing

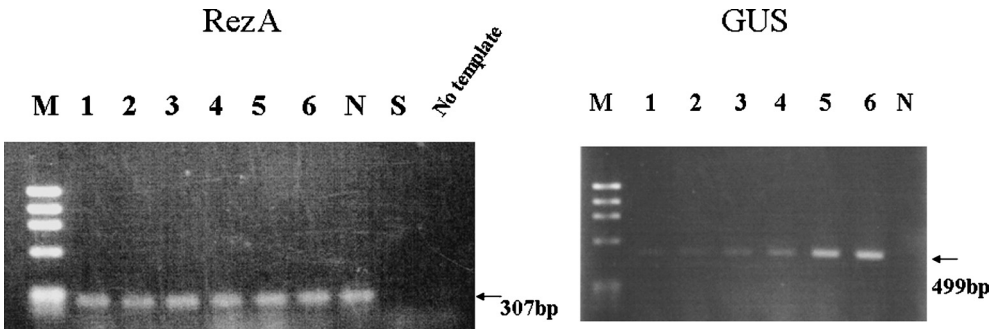

Figure 5. RT-PCR analysis of transgenic calli transformed with pPZP-4×E-RezA135-GUS and pPZP-8×E-RezA135-GUS. *RezA* gene expression is indicated as a 307-bp band corresponding to the 3′-region of the *RezA* gene transcript. *GUS* gene expression is indicated as a 499-bp band corresponding to the 3′-region of the *GUS* gene transcript. Samples are transgenic calli (lanes 1–6), non-transformed calli (N), non-transformed shoot (S), and negative control (No template). For the negative control, PCR was performed without template in the PCR mixture.

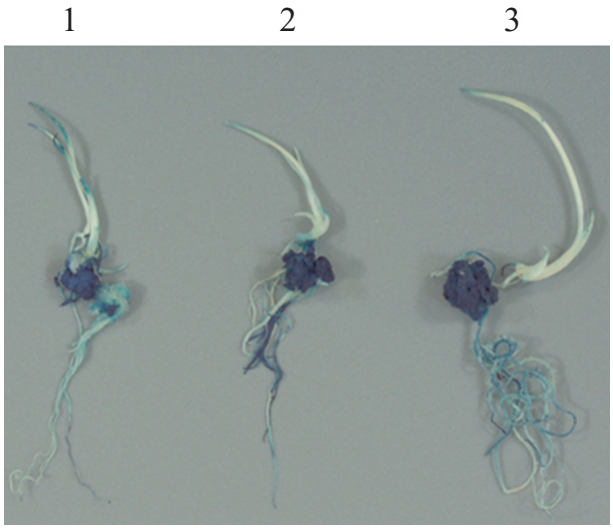

Figure 6. Expression of *GUS* gene driven by the chimeric promoter in transgenic plants at early regeneration stage. T_0 transgenic plants were stained for GUS. Samples are plants transformed with pPZP-4xE-RezA135-GUS (1) and pPZP-8xE-RezA135-GUS (2, 3).

genes into rice.

Histochemical analysis of activity of the chimeric promoter in transgenic rice plants

To examine the mode of activity of the chimeric promoter, we looked at histochemical GUS staining of transgenic calli and plants that were transformed with the same vectors as those in Figure 5, namely pPZP-4xE-RezA135-GUS and pPZP-8xE-RezA135-GUS. As shown in Figure 6, strong GUS activity was detected in the callus, and weaker activity was detected in roots and shoots. We presumed the positive GUS staining in the roots and shoots to be due to the effect of the tandem repeats of the enhancer from the CaMV35S promoter. The positive GUS staining in roots raises the possibility that regenerating roots may be resistant to hygromycin B if the chimeric promoter:*HPT* gene is introduced. In fact, the regenerating callus carrying the chimeric promoter:*HPT* gene could be maintained on the regeneration medium containing hygromycin B and developed normally (data not shown). When *HPT*

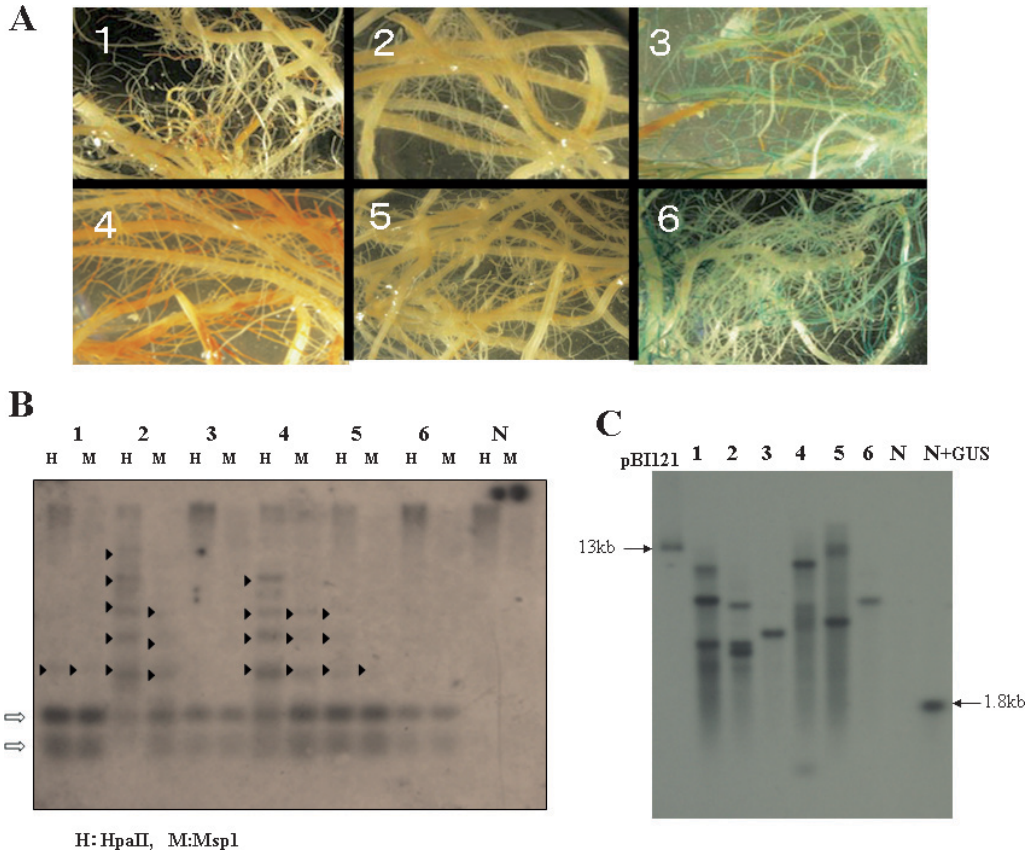

Figure 7. Expression, DNA methylation, and copy number of integrated *GUS* gene in the transgenic plants. Samples 1, 2, 3 are plants transformed with pPZP-4xE-RezA135-GUS, and samples 4, 5, 6 are transformed with pPZP-8xE-RezA135-GUS. (A) Histochemical examination of *GUS* gene expression in roots of transgenic plants. (B) Analysis of DNA methylation in the transgenic plants in Figure 7A. The blot was hybridized with labeled *GUS* gene probe. *Hpa*II (H), *Msp*I (M), and non-transformed control DNA (N). White arrows show bands produced by digestion of unmethylated *GUS* gene. Black arrowheads denote undigested (methylated) DNAs. (C) Southern analysis for estimation of copy number of the integrated *GUS* gene in the transgenic plants in Figure 7A. The blot was hybridized with labeled *GUS* gene probe. As a single-copy reconstruction standard, 162.5 pg of the pBI121 plasmid (pBI121) and 22.5 pg of the *GUS* gene probe mixed with 10 μg of the genomic DNA from non-transgenic rice (N+GUS) were used.

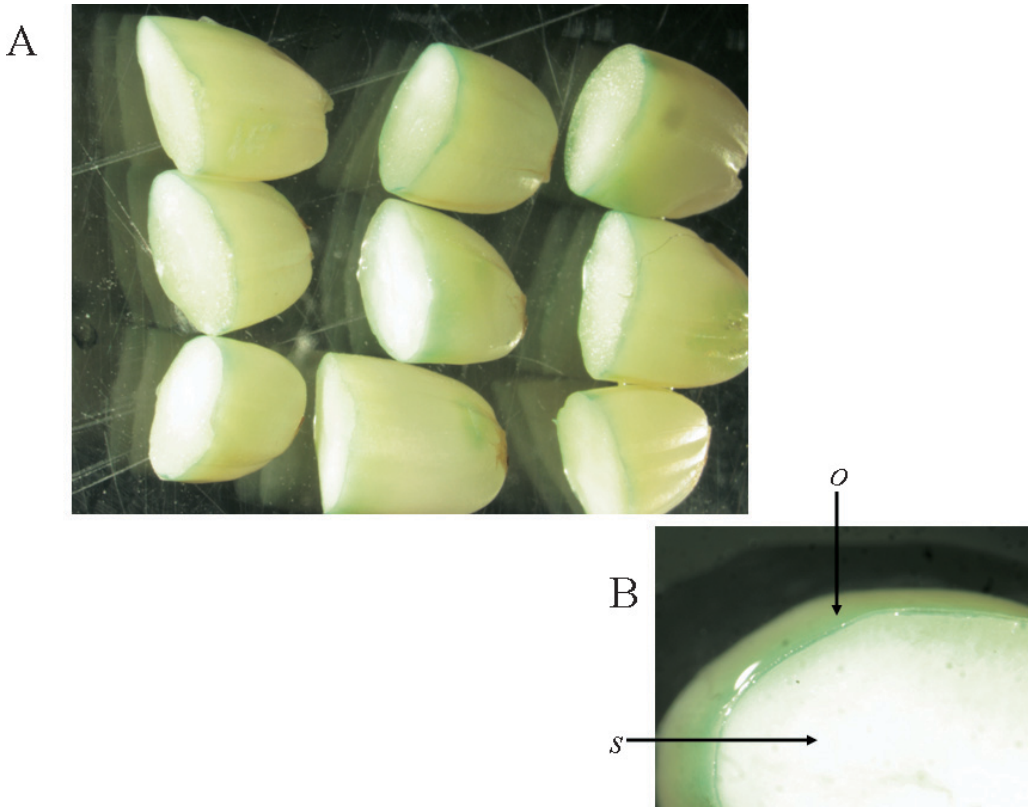

Figure 8. Localization of GUS activity in transgenic rice grains that carried pPZP-4xE-RezA135-GUS or pPZP-8xE-RezA135-GUS. (A) Each seed was obtained from a plant originating from an independent clone, and grains were cross-sectioned before staining. (B) Close-up of stained seed showing GUS activity in the outer layer of the starch storage tissue. *s*, Starch-filled parenchyma cells. *o*, Outer layer composed of aleurone layer, testa, and pericarp.

expression is controlled under a promoter that is active only in the callus, all the regenerating plants derived from transgenic calli become less able to proliferate on selection medium (Huang et al. 2001), because the roots are not resistant to hygromycin B. For practical use, it is desirable to maintain transgenic plants without any loss during the regeneration phase on the selection medium. Therefore, the chimeric promoter may be more useful than the other callus-specific promoter (Huang et al. 2001).

We found, however, that some of the transformants had roots with no GUS activity (Figure 7A, samples 1,2,4,5). As shown in Figures 7B and C, Southern analysis indicated that the *GUS* gene was integrated into the genomic DNA of all the plants with no *GUS* gene expression in the roots.

We then examined DNA methylation of the *GUS* gene by DNA gel blot analysis with the methylation-sensitive restriction endonucleases *Hpa*II and *Msp*I. *Hpa*II cleaves CCGG sites only when the C residue is not methylated, whereas *Msp*I cleaves C5mCGG but not 5mCCGG. There are many CCGG sites in the *GUS* gene, about every 100 or 200 nucleotides, and therefore if any C residue was not methylated, only two major bands corresponding to about 100 or 200 bp were detected at the bottom of the gel (shown by two white arrows), as shown in Figure 7B, samples 3, 6. On the other hand, as shown in Figure 7B, samples 1, 2, 4, 5, some longer-than-expected bands (shown by black arrowheads) indicate that some C residues in the *GUS* gene were methylated. Thus, the analysis of DNA methylation showed that the *GUS* gene was methylated in the plants with no *GUS* expression in roots (Figures 7A, B, samples 1, 2, 4, 5) but not in the plants with blue-stained roots (Figures 7A, B, samples 3, 6). In higher plants, repeat-induced gene silencing is associated with DNA methylation (Selker 1999; Hirochika et al. 2000; Chan et al. 2004).

In Figure 7C, we estimated the copy number of the integrated *GUS* gene from the band in the single-copy reconstruction lane. As a single-copy reconstruction standard, we used the pBI121 plasmid digested with *Hin*dIII (13 kb) and the PCR product of *GUS* (1.8 kb). The number of copies per genome in each transgenic plant was estimated from both 162.5 pg of pBI121 and 22.5 pg of the *GUS* gene, corresponding to a single copy per rice genome. The densities of the bands from samples 3 and 6 were intermediate between the two standard lanes. These results show that the integrated copy number was 1 in samples 3 and 6. Plants with

unstained roots (Figures 7A, C, samples 1, 2, 4, 5) had multiple copies of the *GUS* gene.

Then we examined the expression pattern of the chimeric promoter in the rice grains. As can be seen in Figures 8A and B, GUS was not detected in the starch storage parenchyma, only in the surface layer. Brown rice is usually milled to remove the surface layer, which is composed of pericarp, testa, and aleurone layers, because these are hard to digest and spoil the taste. These results indicate that polished rice endosperm (edible tissue) free from marker gene products could be obtained by using the chimeric promoter for transformation. In the face of public perception about the lingering presence of marker gene products in foods, the absence of such products in edible tissue could be beneficial for commercialization of transgenic rice.

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