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Identification of Regulatory Elements Involved in Expression of a Rice Endopeptidase Gene *Rep1* in Embryos

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Abstract

A gene for a cysteine endopeptidase REP-1, *Rep1*, is expressed in both the embryo and aleurone layers in germinating rice seeds. The expression of *Rep1* was not repressed in the embryo by exogenously applied uniconazole, a gibberellin (GA) biosynthesis inhibitor, even though GA-inducible expression of the gene occurred in the aleurone layers. Serially truncated *Rep1* promoters fused to the β-glucuronidase (GUS) reporter gene were employed to identify the *cis*-regulatory elements involved in the embryo-specific expression. The deletion of the region from -500 to -432 decreased the GUS activity significantly. Linker-scan mutation analysis was carried out to determine the sequences involved in the expression of *Rep1* in embryos, which showed that the region from -500 to -432 contained at least two positive regulatory elements.

Key words: embryo, endopeptidase, gibberellin, promoter analysis, rice.

Abbreviations

ABA, abscisic acid; CaMV, cauliflower mosaic virus; EDTA, ethylenediaminetetraacetic acid; GA, gibberellin; GARE, GA-response element; GUS, β-glucuronidase; Luc, luciferase.

Introduction

Cereal grains such as rice, barley and wheat, store most of their reserves, those are principally starch and proteins, in endosperms surrounded by aleurone layers where hydrolases are synthesized (Bewley and Black, 1994). During seed germination and early seedling growth, the storage protein in the endosperm is digested by endopeptidases secreted from the aleurone layers and is mobilized to supply amino acids that support the growth of the embryo. The synthesis of the endopeptidases in aleurone layers is induced by gibberellin (GA) and this induction is antagonized by abscisic acid (ABA). Expression of the gene for a barley cysteine endopepetidase EP-B, *EPB1*, in aleurone layers has been induced by GA (Koehler and Ho, 1990). Analysis of the *EPB1* promoter indicated that the GA-response element (GARE), TAACAG/A, conserved in cereal GA-inducible genes was required for the GA-inducible expression (Cercós *et al.*, 1999).

We previously purified a cysteine endopeptidase REP-1 (rice endopeptidase-1) from rice seedlings (Kato and Minamikawa, 1996). The enzymatic characterization of REP-1 revealed that this enzyme mainly plays a role in the degradation of the storage protein glutelin, which accounts for about 80% of the total protein in rice seeds. A cDNA clone for REP-1, named pRP60, was isolated and its sequence indicated that REP-1 belonged to a papain-like cysteine proteinase (Kato and Minamikawa, 1996). We isolated a clone for the REP-1 gene, *Rep1*, pgRP60, and its nucleotide sequence showed that the gene is intron-less (Kato *et al.*, 1999). We also isolated a cDNA clone encoding another papain-like cysteine proteinase named pRP80 and isolated a clone for the corresponding gene, *RepA* (Shintani *et al.*, 1995; Kato *et al.*, 1999). Northern blot analysis showed that mRNAs for both *Rep1* and *RepA* were undetectable in dry seeds, but their levels increased sharply upon imbi-

bition (Shintani *et al.*, 1997). When the aleurone layer was incubated without hormone, mRNAs for the two proteinases were maintained at a low level. However, the levels of the two mRNA in this tissue were increased by the addition of GA_3, and the effect was eliminated by the addition of ABA. GA synthesized in the embryo is secreted to the aleurone layers and induces hydrolase gene expression (Kaneko *et al.*, 2002). The *RepA* mRNA level in seedlings was reduced by exogenously applied uniconazole, a GA biosynthesis inhibitor, but this inhibitor had a weak effect on the repression of *Rep1* expression. We presumed that GA was not required for the expression of *Rep1* in rice embryos.

It is known that GA induces α-amylase expression in the aleurone layers of cereal seeds (Bewley and Black, 1994), but Umemura *et al.* (1998) found that α-amylase expression in rice embryos was regulated by sugar starvation: the expression of *RAmy3D* in the embryos was repressed by exogenously applied sugar. Using a transient expression system with particle bombardment to analyze the sugar-sensing mechanism of the *RAmy3D* promoter activity, Toyofuku *et al.* (1998) identified the promoter elements required for sugar-induced repression of the *RAmy3D* in rice embryos, and these sequences are different from GARE. These reports indicate that the regulation of α-amylase expression is different in embryos and aleurone layers.

In the present study, we used uniconazole, a triazole-type growth retardant which inhibits the biosynthesis of GAs at the conversion of *ent*-kaurene to *ent*-kaurenoic acid (Izumi *et al.*, 1985), to show that GA was not required for *Rep1* expression in rice embryos. The promoter region of *Rep1* was analyzed with a transient assay system to identify the *cis*-regulatory elements in the embryos. Our results from the deletion and linker-scan analyses showed that the region from -500 to -432 was required for the high-level expression of *Rep1* and included at least two positive regulatory elements.

Materials and Methods

Plant materials

Rice seeds (*Oryza sativa* L. cv. Nipponbare) were kindly gifted to us by Dr. Seiichiro Kiyota (National Institute of Agrobiological Resources, Tsukuba, Japan). The rice seeds were husked and surface-sterilized by treatment with 1% Antiformin including 0.05% Tween 80 for 60 min followed by thorough washing with sterile water. The seeds were allowed to germinate at 27 ℃ in darkness for 5 days in the presence or the absence of 50 μM uniconazole. For the preparation of rice embryos, the rice seeds were germinated in liquid medium (Murashige and Skoog medium with 3% sucrose and 2.2 mg l^{-1} 2,4-dichlorophenoxyacetic acid) as described by Umemura *et al.* (1998). After incubation for seven days, the embryos were dissected from the endosperms, and transferred to the same liquid medium as described above.

Isolation of total RNA and Northern blot analysis

Total RNA was prepared as described by Karrer *et al.* (1991). The glyoxylated RNAs were separated by electrophoresis on a 1.4% agarose gel and were blotted onto a Hybond-N nylon membrane (Amersham Biosciences). A cDNA clone for *Rep1*, pRP60, was used as the probe for *Rep1*. To probe α-amylase mRNA, the partial cDNA clone for rice α-amylase corresponding to the region from +549 to +1268 of *RAmy1A* cDNA was cloned as described previously (Karrer *et al.*, 1991). ^{32}P labeling of cDNA fragments was conducted with a random primer DNA labeling kit (Takara Shuzo). Hybridization was performed in a plastic bag filled with 50% formamide and 5x SSPE (1x SSPE: 0.5 M NaCl, 10 mM $NaHPO_4$, 1 mM EDTA, pH 7.4) for 16 h at 42 ℃.

Construction of reporter plasmids

The truncated fragments of the *Rep1* promoter were amplified by polymerase chain reaction (PCR) and two mutations for creating *Hin*dIII and *Spe*I sites were simultaneously generated at the 5' and the 3' ends of the fragments, respectively. The amplified fragments were cloned to pCR 2.1 vector (Invitrogen) to confirm the sequences, and then cut from the plasmids with *Hin*dIII and *Spe*I, re-inserted into the β-glucuronidase (GUS) vector, pBI221 (Jefferson *et al.*, 1987), in the replacement of the cauliflower mosaic virus (CaMV) 35S RNA promoter to construct RX-184, RX-367, RX-432, RX-500, RX-568 and RX-808. Several point-mutated constructions were generated from RX-500 by PCR-based oligonucleotide-directed mutagenesis (Picard *et al.*, 1994) with 6-bp point mutations and 9-bp intervals. The amplified fragments were inserted into the pBI221 vector as described above. Nucleotide sequences of the amplified fragments were confirmed with an automatic DNA sequencer (Prism model 310, PE Applied Biosystems).

Delivery of DNA-coated gold particles into rice embryos

Gold particles (1 μm in diameter; Tokuriki Honten) were conjugated with DNA at 7 μg mg^{-1} (DNA per particles) by ethanol precipitation. The

luciferase (Luc) reporter plasmid pAHC18 (Christensen and Quail, 1996) was always introduced into the rice embryos as an internal control. Fifty micrograms of the gold particles were bombarded into five embryos at a helium gas pressure of 4 kgf cm^{-2} with a biolistic device (IDERA GIE-III, Tanaka). The distance between target tissue and the nozzle of the gun was fixed at 4 cm. After bombardment, five embryos were incubated in 5 ml of shooting buffer (20 mM sodium succinate pH 5.5, 20 mM $CaCl_2$ and 10 μg ml^{-1} chloramphenicol) in a petri dish with shaking for 20 h at room temperature. Ten embryos were then homogenized using a mortar and a pestle in 200 μl of the extraction buffer containing 100 mM KPO_4, 2 mM 1,4-dithiothreitol, 2 mM EDTA and 5% glycerol (pH 7.8). The homogenate was centrifuged at 12,000 *g* for 30 min at 4 ℃ and the supernatant was retained for measurement of the activities of GUS and Luc. In the Luc assay, 20 μl of each extract was reacted with a commercial kit (Pica Gene, Toyo Ink). The peak light intensity was measured using a luminometer (NU-600, Nichion). In the GUS assay, 50 μl of each extract was reacted with 4-methylumbelliferyl-β-D-glucuronide as a substrate according to Kosugi *et al.* (1990). The GUS activity was measured in a spectrofluorophotometer (RF-5000, Shimadzu). The relative GUS activity was normalized against the Luc activity.

Results and Discussion

Rep1 expression in rice embryo

The expression of *Rep1* in the aleurone layers was induced by GA (Shintani *et al.*, 1997). Uniconazole was exogenously applied to rice seedlings. However, uniconazole did not effect the levels of *Rep1* mRNA in the seedlings (**Fig. 1**). The expression of *Rep1* was found in rice embryos and its level was not affected by exogenously applied uniconazole. Previously, *RAmy1A*, a rice α-amylase gene, was shown to be expressed in embryos (Itoh *et al.*, 1995). Therefore, we examined the effect of exogenously applied uniconazole on the expression of *RAmy1A* in rice embryos and found that it did not completely suppress the *RAmy1A* expression in seedlings and had only a weak effect on the repression of *RAmy1A* expression in the embryo (**Fig. 1**).

GA-induced α-amylase gene expression in the aleurone layers of barley seeds has been shown to be unaffected by the presence of sugar, whereas exogenously applied sugars did effect effect α-amylase gene expression in embryos (Perata *et al.*, 1997). Exogenously applied uniconazole decreased the endogenous GA level in the embryos and reduced *RAmy1A* mRNA to 30% (Loreti *et al.*, 2000). In rice embryos, uniconazole decreased the *RAmy1A* mRNA level slightly but did not affect the *Rep1* mRNA level (**Fig. 1**). This result suggests that GA is not required for the expression of *Rep1* in the embryo, and that the mechanisms of expression are different in embryos and aleurone layers. Ho *et al.* (2000) isolated the gene for *OsEP3A* corresponding to *Rep1* and this promoter region fused to the GUS reporter gene was introduced into rice suspension-cultured cells. The promoter activity of *Rep1* was increased by nitrogen starvation.

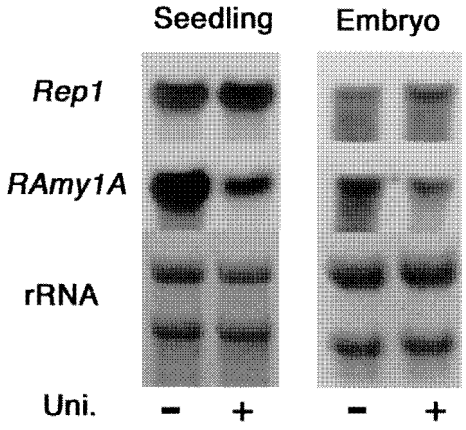

Fig. 1 Effects of a GA biosynthesis inhibitor, uniconazole, on the gene expression of proteinase (*Rep1*) and α-amylase (*RAmy1A*). Total RNA (10 μg per lane) was analyzed by Northern blotting with cDNAs for *Rep1* and *RAmy1A* as probes. Seedlings (Seedling) or embryos (Embryo) were incubated in the presence (+) or the absence (-) of 50 μM uniconazole (Uni.). For the detection of rRNA, the gel was stained with 0.04 % methylene blue in 0.5 M sodium acetate buffer at pH 5.2.

We examined the effect of nitrogen on *Rep1* expression with a transient assay system described below. The reporter construct RX-500, including *Rep1* promoter fused to the GUS gene coding region, was introduced into rice embryos which were incubated in medium with or without a nitrogen resource, but the presence or absence of nitrogen in the medium did not affect *Rep1* promoter activity (data not shown). The factors involved in *Rep1* expression in rice embryo remain to be defined.

Deletion analysis of the Rep1 promoter

To delineate the region involved in *Rep1* expression, we constructed six reporter plasmids derived from pBI221 in the replacement of CaMV 35S RNA promoter with the truncated *Rep1* promoter. When the *Rep1* promoter was truncated to -808 or -568,

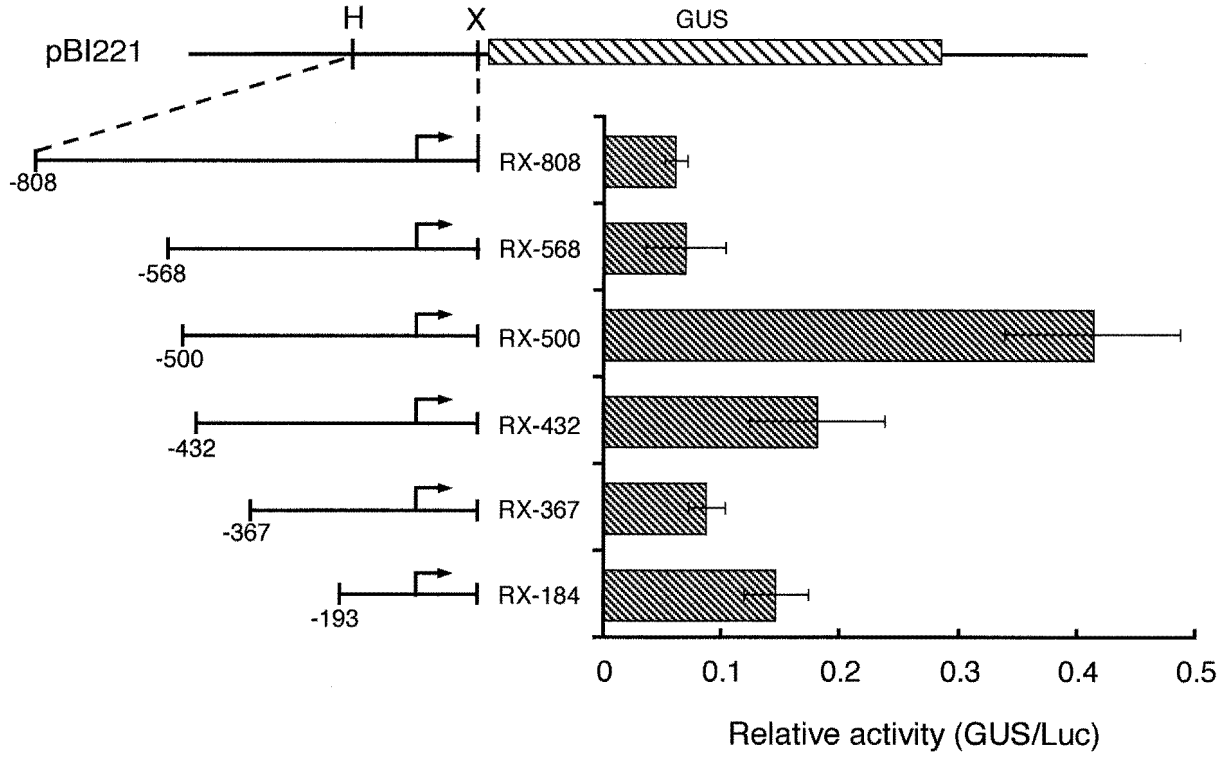

Fig. 2 Deletion analysis of the *Rep1* promoter region. Reporter constructs carry a series of truncated promoter regions of *Rep1*. After bombardment, the rice embryos were incubated for 24 h. Then the enzymatic activities were measured as described in the "Materials and Methods section". The restriction enzyme recognition sites of *Hin*dIII and *Xba*I are indicated by H and X in pBI221, respectively. Arrows indicate directions of the transcription of *Rep1*. Relative activity was calculated by dividing the GUS to Luc ratio from each construct by the GUS to Luc ratio obtained from pBI221. The results shown are means and standard errors from four independent bombardments.

GUS activities remained at low levels (**Fig. 2**). However, the *Rep1* promoter truncated to -500 allowed five-fold higher GUS activity compared with those from the above two constructs. Further deletion of the *Rep1* promoter reduced the GUS activity. These results suggest that the negative and positive regulatory elements are located in the region from -568 to -500 and from -500 and -432, respectively.

Shintani *et al.* (1997) showed that GA and ABA regulate the expression of *Rep1* in aleurone layers. Recently, we found that two GAREs that exist in the region downstream -184 of the *Rep1* promoter were involved in GA-inducible expression in the aleurone layers (unpublished data). Thus, it is possible that the regulation of *Rep1* expression differs in embryos and aleurone layers in germinating rice seeds.

Identification of the positive regulatory element by linker-scan mutation

To identify the *cis*-regulatory elements in the region from -500 to -432, point mutations were generated from RX-500 as described in **Fig. 3A.** When RX-500lm-1 and RX-500lm-2 were bombarded, the GUS activities were reduced significantly compared with the original plasmid RX-500 (**Fig. 3B**). However, the GUS activity of RX-500lm-3 was equal to that of the original construct. These results indicate that the sequence from -492 to -476 contributes to *Rep1* expression in the embryos. Furthermore, the point mutations in RX-500lm-4, RX-500lm-5 and RX-500lm-6 showed reduced GUS activities, whereas the point mutation in RX-500lm-7 did not have any effects on the GUS expression. This indicates that the sequence from -465 to -442 is also involved in *Rep1* expression. The fluctuating profile of GUS reporter gene expression as shown in **Fig. 3B** indicates that the region from -432 to -500 contains at least two positive regulatory elements.

We searched for a putative transcription factor binding sequence using the Signal Scan program (Higo *et al.*, 1999) in the *Rep1* promoter region from -500 to -432. As shown in **Fig. 4**, the region from -500 to -432 contains a Myb binding site, CGGTTC (Urao *et al.*, 1993); the core sequence of low-temperature-responsive element, CCGAC

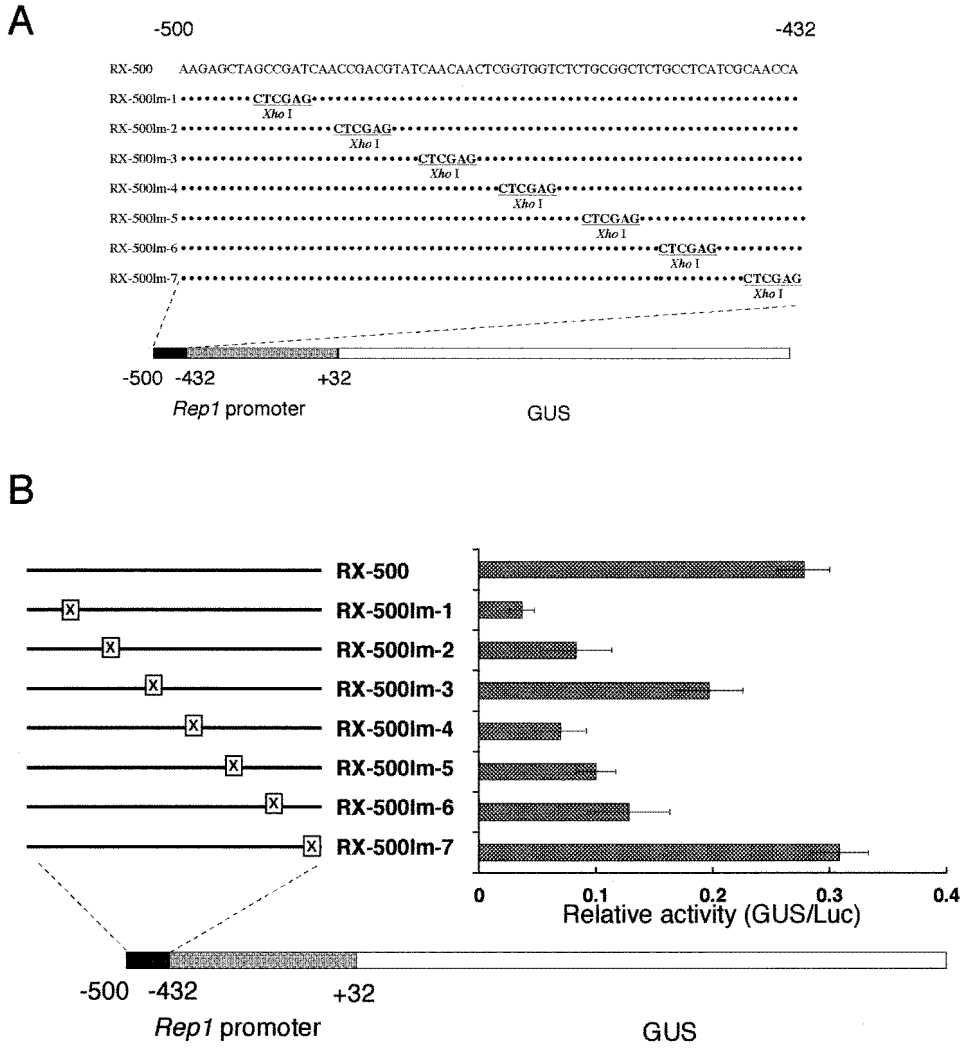

Fig. 3 Linker-scan analysis of the region from -500 to -432 of *Rep1* promoter. (A) The nucleotide sequence of the wild-type *Rep1* is shown in the top lane (RX-500) and the successive point mutation sequences are shown in the lanes below (RX-500lm-1 to RX500lm-7, respectively). Mutated nucleotide sequences are underlined. Dots represent unaltered nucleotides. A schematic diagram of the forefather construct, RX-500, is shown at the bottom of the constructs. (B) Models of linker-mutated constructs are shown on the left. A schematic diagram of the forefather construct, RX-500, is shown at the bottom of the constructs. A boxed X indicates the point of mutation. Relative activity was calculated by dividing the GUS to Luc ratio from each construct by the GUS to Luc ratio obtained from pBI221. The results shown are means and standard errors from three or more independent bombardments.

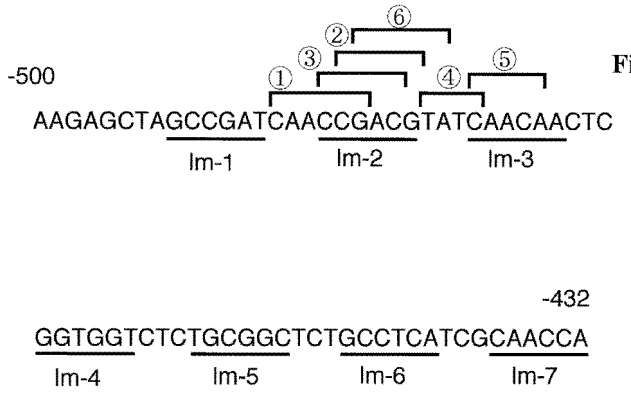

Fig. 4 The putative regulatory elements in the *Rep1* promoter region from -500 to -432. Underlining indicates the point mutation sequences of the constructs used for the linker-scan analysis. The following sequences are indicated by numbers in blankets: 1, Myb binding site; 2, low-temperature-response element; 3, CGACG element; 4, GATA box; 5, RAV1 binding site; and 6, bZIP binding site.

(Baker *et al.*, 1994); a CGACG element (Hwang *et al.*, 1998); a GATA box (Lam and Chua, 1989); and a RAV1 binding site, CAACA (Kagaya *et al.*, 1999). Moreover, we also found a GACGTA sequence to which the bZIP transcription factor bound (Williams *et al.*, 1992). These sequences are located between -495 to -469. We could not find any reported *cis*-regulatory elements in the region from -465 to -442 involved in *Rep1* gene expression. The sequence between the point mutation sites of RX-500lm-1 and RX-500lm-2 contains four putative regulatory elements. The low-temperature-responsive element is excluded in this report, because we focused on the embryo-specific expression of *Rep1*. The CGACG element is involved in sugar-starvation induction of the rice α-amylase gene, *RAmy3D*, in embryos (Hwang *et al.*, 1998). However, this element may not be involved in *Rep1* expression in the embryos, because sugar-starvation did not induce *Rep1* expression (Ho *et al.*, 2000). A Myb transcription factor, GAMyb, is known to be involved in the GA-inducible expression of genes for α-amylase and proteinase in aleurone layer cells (Gubler *et al.*, 1995). But the GARE TAACAG interacting with GAMyb, is different from the Myb binding sequence in the region from -500 to -432 in the *Rep1* promoter. Because Myb transcription factors are encoded by a multigene family (Suzuki *et al.*, 1997), *Rep1* expression in embryos may be regulated by Myb transcription factors differently from GAMyb. The sequence GACGTA, recognized by bZIP transcription factors (Williams *et al.*, 1992), is found in the promoters of the genes for cysteine proteinases from leguminous plants (Akasofu *et al.*, 1990; Ogushi *et al.*, 1992). But these promoters are not well characterized, although the two genes have been shown to be expressed in the embryos of germinating seeds (Yamauchi *et al.*, 1992; Tanaka *et al.*, 1993). It is possible that the GACGTA motif is involved in the expression of proteinases in the embryos of germinating seeds.

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