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The *rab16B* Promoter of Rice Contains Two Distinct Abscisic Acid-Responsive Elements

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To localize abscisic acid (ABA)-inducible gene expression of *rab16* genes, *rab16A* promoter was linked to the *gusA* reporter gene encoding β-glucuronidase and introduced into rice (*Oryza sativa* L.) plants. The activity of *rab16A* promoter was induced by ABA and osmotic stresses in various tissues of vegetative and floral organs. In anthers and embryos, *rab16A* promoter was active in the absence of ABA. To elucidate *cis*-elements of the *rab16* promoter that confer ABA-inducible expression, variously modified 40-bp fragments (−264 to −225) of the *rab16B* promoter were fused to a truncated (−46 bp) cauliflower mosaic virus 35S minimal promoter, and their activities in protoplasts were analyzed. The transient assays revealed that the 40-bp fragment consists of two separate ABA-responsive elements, motif I (AGTACGTGGC) and motif III (GCCGCGTGGC). Motif I and motif III are both required for ABA induction; however, each can substitute for the other. Further analyses of these motifs indicated that motif III has a distinct DNA sequence specificity as an ABA-responsive element from motif I, suggesting that the two motifs interact with different transcription factors in vivo.

It is established that ABA mediates gene expression involved in seed maturation and tolerance against physiological stresses such as desiccation and high-salt conditions (Skriver and Mundy, 1990; Hetherington and Quatrano, 1991). To understand the molecular mechanisms of gene regulation exerted by ABA, a number of genes responsive to ABA have been isolated and characterized in a variety of plant species (for review, see Skriver and Mundy, 1990; Giraudat et al., 1994). The *rab16* gene family of rice (*Oryza sativa* L.) consists of four tandemly arrayed genes, *rab16A* to *rab16AD*, which are highly expressed in embryos during the late stage of seed development, and which respond to ABA and water stress in vegetative tissues (Mundy and Chua, 1988; Yamaguchi-Shinozaki et al., 1989).

A comparison of the promoter sequences of the four *rab16* genes revealed two highly conserved motifs, motif I (5′-GTACGTGGC-3′) and motif II (5′-CGG/CCGCGCT-3′) (Yamaguchi-Shinozaki et al., 1989). Previous footprinting experiments revealed that these two conserved sequences were bound by nuclear proteins (Mundy et al., 1991). Transient expression experiments using barley aleurone protoplasts showed that, when fused with the −90-bp CaMV 35S promoter, a hexamer of motif I conferred ABA-inducible gene expression, whereas motif II did not, suggesting that motif I is an essential part of the ABRE (Skriver et al., 1991). The expression pattern of the wheat *Em* gene (Williamson and Quatrano, 1988) is similar to that of the rice *rab16* genes. The *Em* promoter also possesses two highly conserved boxes, *Em1a* (CACGTGGC) and *Em1b* (ACACGTGCC), which are highly homologous to motif I of the *rab16* genes (Marcotte et al., 1989). Transient assays using rice protoplasts identified a 50-bp fragment (−152 to −103) containing the two highly conserved boxes as an ABRE (Marcotte et al., 1989). Further analysis of the *Em* promoter that has a 2-bp mutation in the *Em1a* indicated that *Em1a* is indispensable for ABA induction of the *Em* gene (Guiltinan et al., 1990). In addition, *Osvp1* (Hattori et al., 1994), a rice homolog of the maize transcriptional activator *Viviparous-1* (*Vp1*) (McCarty et al., 1991), has been shown to regulate gene expression of a rice ABA-inducible gene, *Osem*, which is a homolog of the wheat *Em* gene. It is interesting that the *cis*-element necessary for both ABA induction and *Osvp1* activation of the *Osem* gene was identified as a motif-I-like element (Hattori et al., 1995). Recently, Vasil et al. (1995) reported that both *Em1a* and *Em1b* of the wheat *Em* promoter are sufficient to confer the *Vp1* transactivation and the synergistic interaction with ABA in maize protoplasts. These studies suggest that motif-I-like elements play a major role in ABA-inducible gene expression in various species.

On the contrary, there are *cis*-acting elements that do not resemble motif I but are involved in ABA induction. A maize ABA-inducible gene, *C1*, was shown to be regulated by the maize *Vp1* (McCarty et al., 1991) gene. The *cis*-acting element involved in both ABA induction and *Vp1*-mediated activation was identified as GGTCGTGTGGTCCATGCATGCAC (the underlined sequence is known as a *Sph*I element), which does not resemble motif I (Hattori et al., 1992). Apparently, this result is different from that of *Em1*

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Abbreviations: ABRC, ABA-responsive complex; ABRE, ABA-responsive element; b-ZIP, basic region–leucine zipper; CaMV 35S, cauliflower mosaic virus 35S; CE, coupling element; X-gluc, 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid.

(Vasil et al., 1995), suggesting that two distinct elements, a motif-I-like element and an *Sph*I element, are involved in ABA-inducible gene expression. Furthermore, recent studies have revealed that other elements distinct from motif I are also involved in ABA-inducible gene expression of the barley *HVA1* gene (Straub et al., 1994), the barley *HVA22* gene (Shen et al., 1993), the sunflower *Ha*G3-A gene (Nunberg et al., 1994), and the *Craterostigma plantagineum CDeT*27-45 gene (Nelson et al., 1994). Recent analyses of the barley *HVA22* promoter (Shen and Ho, 1995) revealed that ABRC1, composed of ABRE3 (GCCACGTACA, motif-I like) and CE1 (TGCCACCGG), is sufficient for high-level ABA induction. Taken together, two classes of *cis*-elements unrelated to motif I are involved in ABA-inducible gene expression. The first class includes CEs such as CE1 of the barley *HVA22*, which associates with motif-I elements. The second includes elements such as the *Sph*I box of the maize *C1*, which can act as an ABRE alone. Functions of these elements distinct from motif-I-like elements in ABA-inducible expression remain to be characterized in detail.

In this investigation we first introduced the *rab16A*/GUS gene into rice plants and examined the activity in various tissues of transgenic rice. Using substitution mutation analyses, we next discovered that a 40-bp fragment of the *rab16B* promoter consists of two separate ABREs, motif I (AGTACGTGGC) and motif III (GCCGCGTGGC).

MATERIALS AND METHODS

Transformation of Rice Plants

Transformation of rice plants (*Oryza sativa* L. cv Toride 1) was carried out as previously described (Shimamoto et al., 1989). The *rab16A*/GUS plasmid was co-transformed into rice protoplasts with the hygromycin phosphotransferase gene as a selection marker. Introduction of the *rab16A*/GUS was verified by staining a portion of the calli with X-gluc. From GUS-positive calli, transgenic plants carrying the *rab16A*/GUS gene were regenerated. Primary transformants were self-fertilized to obtain seeds.

Plasmid Constructions

All plasmids were constructed using a standard recombinant DNA technique (Sambrook et al., 1989). All plasmids in this work were derivatives of pIG221, which contains both the initiation site (ATG) and the first intron of the castor bean catalase gene and is translationally fused to the *gusA* gene (Tanaka et al., 1990). As a first step, the 0.8-kb *Hin*dIII-*Xba*I fragment of pIG221, which contains a full-length CaMV 35S promoter, was substituted with a 0.1-kb *Hin*dIII-*Xba*I fragment, which contains a truncated CaMV 35S (−46 bp) promoter, to yield pIG46. This 0.1-kb fragment was obtained after the *Xho*I-*Pst*I fragment of X-GUS-46 was subcloned into the vector (pBluescript SKII$^-$, Benfey et al., 1990). The resulting pIG46 has a *Hin*dIII and a *Xho*I site at the upstream region of the truncated 35S promoter. All of the 70-bp, 40-bp, and tetramer DNA fragments tested were synthesized by a DNA synthesizer (Applied Biosystems) and introduced between the two restriction sites of pIG46. All plasmids were verified using a DNA sequencer (Applied Biosystems).

Genomic DNA Blot Analysis

Preparation of genomic DNA from transgenic rice plants and Southern blot analysis were carried out as previously described (Southern, 1975; Shimamoto et al., 1989).

Histochemical Analysis

Transgenic plants about 15 cm in height were incubated in 10^{-4} M ABA or 1% NaCl for 16 to 18 h at 30°C. Flowers were collected from mature plants in a greenhouse, immersed in water in the presence of either 10^{-4} M ABA or 1% NaCl, and kept for 16 to 18 h at 30°C. Mature seeds were kept in water with 10^{-4} M ABA for 16 to 18 h at 30°C. For desiccation treatment, transformants were left on the bench without water for 16 to 18 h at 30°C.

Histochemical staining was performed using a solution containing 1 mM X-gluc and 50 mM sodium phosphate buffer (pH 7.0) as described previously (Jefferson et al., 1987). Mature dry seeds were manually cut into halves with a razor blade. Leaves and roots were similarly cut into thin sections. All of the tissue samples were incubated in the X-gluc-staining solution for 16 to 18 h at 37°C.

Transient GUS Analysis

Protoplasts were isolated from the rice *Oc* suspension cell line (Baba et al., 1986). Protoplasts (4×10^6 cells) were electroporated with 25 μg of test plasmids under the conditions described previously (Izawa et al., 1994). After electroporation, one-half of the protoplasts was cultured in R2P medium with 5×10^{-5} M ABA, and one-half was cultured without ABA for 16 to 18 h at 30°C. The protoplasts were then harvested and fluorometric measurement of GUS activity in protoplast extracts was carried out according to the method of Jefferson et al. (1987). The 4-methylumbelliferyl-β-D-glucuronide solution was modified to include 20% methanol to reduce the background activity (Kosugi et al., 1990). Soluble proteins in extracts were measured using a protein assay kit (Bio-Rad).

RESULTS

The *rab16A* Promoter Responds to Various Signals in Transgenic Rice

To obtain transgenic rice plants carrying the *rab16A*/GUS gene, hygromycin-resistant calli expressing GUS enzymatic activity were screened by staining with X-gluc solution and plants were regenerated from the GUS-positive calli. Because the first intron of the castor bean catalase gene was previously shown to enhance expression of foreign genes in transgenic rice (Tanaka et al., 1990; Hayakawa et al., 1992), we inserted this intron into the *rab16A*/GUS gene (Fig. 1A).

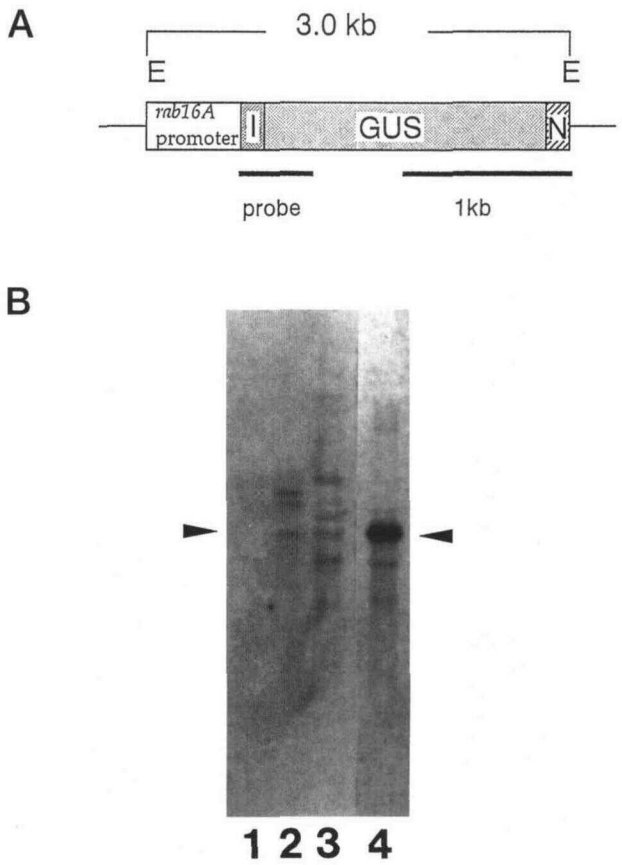

Figure 1. Southern blot analysis of transgenic rice plants carrying *rab16A*/GUS. A, A schematic representation of p*rab16A*/GUS. *rab16A* promoter, An 800-bp region of *rab16A* promoter; I, 190-bp castor bean catalase intron 1, GUS, *Escherichia coli gusA*-coding region; N, nopaline synthase terminator; E, *Eco*RI site; probe, a 382-bp fragment of *gusA*. B, Southern blot analysis of transgenic rice plants. Genomic DNA (1 μg) isolated from independent transgenic plants was digested with *Eco*RI, subjected to electrophoresis in an agarose gel, transferred to a nylon membrane, and allowed to hybridize with the probe. Lane 1, Untransformed control plant; lane 2, transgenic plant 21–3; lane 3, transgenic plant 21–11; lane 4, plasmid p*rab16A*/GUS. Arrowheads indicate expected 3.0-kb fragment derived from p*rab16A*/GUS.

Fifty-eight primary transgenic plants were obtained from 15 independently transformed calli. Regenerated plants derived from five independent calli were used for subsequent experiments. Progeny seeds were obtained by self-pollination of primary transgenic plant 21–4.

Stable integration of the *rab16A*/GUS gene in transgenic rice plants was examined by Southern blot analysis of genomic DNA isolated from three plants. Figure 1B shows results from two of them, in which *Eco*RI-digested DNA was hybridized with a 0.4-kb GUS probe. Whereas no hybridization signal was detected in untransformed plants, transgenic plants contained the expected 3.0-kb fragment, suggesting stable and proper expression of the *rab16A*/GUS gene.

Histochemical localization of GUS activity was carried out to study ABA-inducible expression of the *rab16A*/GUS gene. Figure 2 shows the representative pattern of *rab16A*/GUS gene expression in various tissues. In roots and leaves expression of the *rab16A*/GUS gene was clearly induced in response to 10^{-4} M ABA, 1% NaCl, and desiccation treatments, whereas GUS activity was rarely detected without these treatments. The induced GUS expression was often observed in only the vascular bundles of leaves and roots, but in some transgenic plants all tissues in these organs exhibited high GUS activity. In anthers and glumes, significant induction of GUS expression was observed with ABA, NaCl, and desiccation (Fig. 2), whereas faint GUS activity was detected in anthers without any treatments. Nonuniform staining of glumes after these treatments was often observed. In seeds, GUS activity was detected in mature embryos without ABA treatment and was slightly induced by the application of ABA (Fig. 2). No GUS activity was detected in endosperm regardless of ABA treatment.

Taken together, expression of the *rab16A*/GUS gene in transgenic rice plants is responsive not only to ABA but also to osmotic stresses in various tissues. These observations correlate well with the accumulation of endogenous *rab16A* transcripts in rice plants under various conditions (Mundy and Chua, 1988; Yamaguchi-Shinozaki et al., 1989).

The 40-bp Fragment Containing Motif I Is an ABRE

To determine *cis*-elements responsible for ABA induction, we chose a 70-bp sequence (from −270 to −202) of the *rab16B* promoter, which contained both highly conserved motif I (AGTACGTGGC) and motif II (CGG/CCGCGCT). We fused the 70-bp fragment and a 40-bp fragment (from −264 to −225) lacking motif II to the −46-bp CaMV 35S core promoter. These chimeric promoters are termed RB70 and RB40, respectively, and their activity was tested by transient assays using rice protoplasts. Figure 3 shows the activation of these promoters by ABA treatment. The *rab16A* promoter was included as a positive control for ABA induction (Fig. 3).

Whereas the −46-bp CaMV 35S promoter showed no significant ABA induction, both the 70-bp fragment and the 40-bp fragment fused to the −46-bp promoter showed an approximately 4- to 5-fold induction by ABA. Therefore, the 40-bp fragment, which includes motif I but not motif III, appears to contain *cis*-elements for ABA-inducible gene expression. This suggests that motif II is not involved in ABA induction of the *rab16B* promoter. However, it cannot yet be ruled out that motif II may be involved but is redundant.

The 40-bp ABRE Consists of Two Separate ABA-Responsive Motifs

To gain further insight into *cis*-elements for ABA induction present in the 40-bp fragment, a series of substitution mutations were made in RB40 (Fig. 4). GUS activities of these mutated 40-bp fragments were measured after ABA treatment using rice protoplasts.

ABA induction was abolished in a mutated promoter (Mut 1), which has a 2-bp mutation in the motif I (from −260 to −252), indicating that motif I is required for ABA-

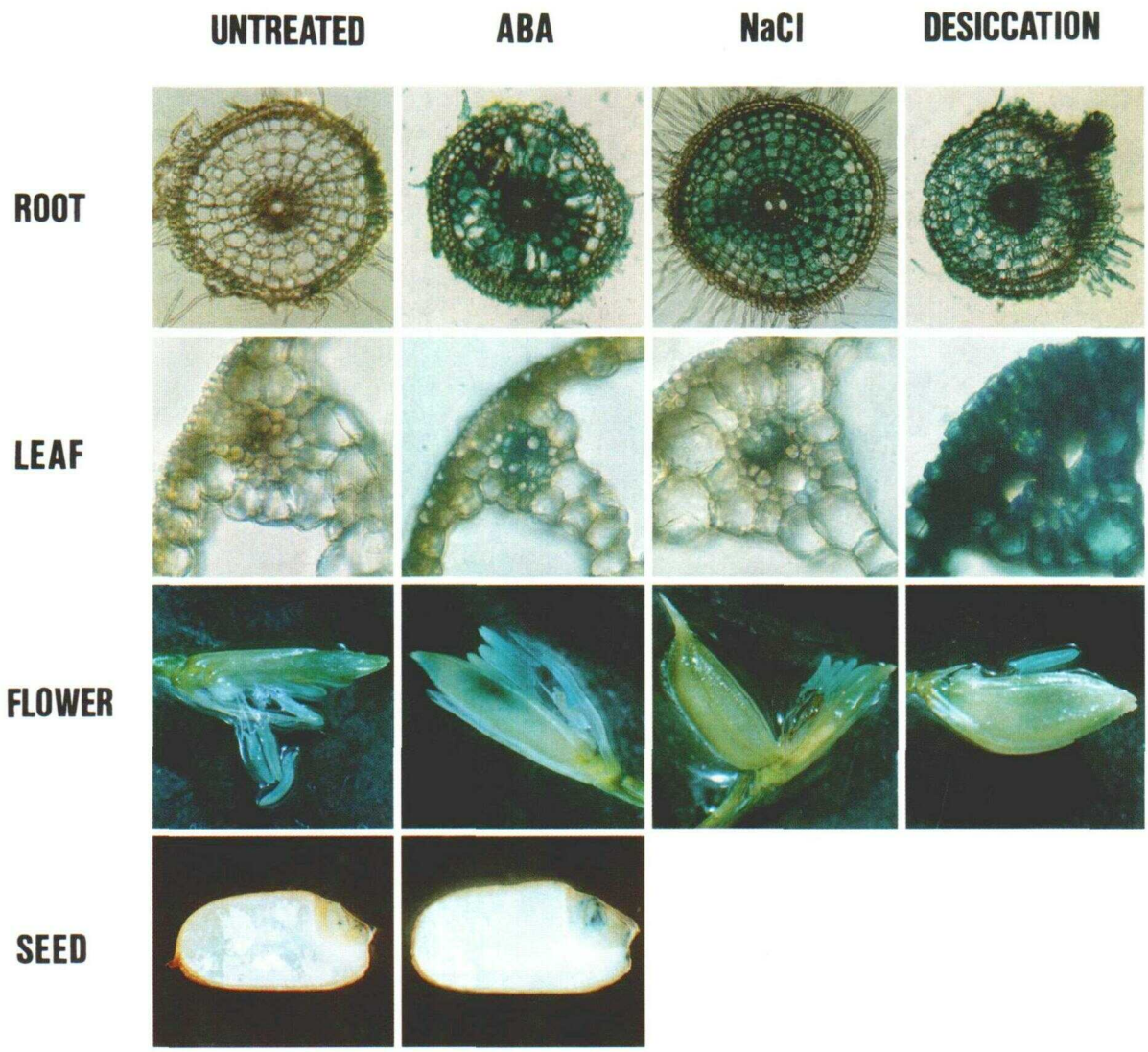

Figure 2. Histochemical localization of *rab16A*/GUS expression in rice. Conditions for various ABA, NaCl, and desiccation treatments are described in "Materials and Methods."

inducible gene expression. Similarly, GUS activity of Mut 3, in which 9 bp from −242 to −234 were mutated, was not induced by ABA treatment. However, Mut 2, which has a 4-bp mutation from −251 to −248, and Mut 4, which has a 9-bp substitution from −233 to −225, did not influence ABA induction (Fig. 4). Therefore, the region between −242 and −234 in the *rab16B* promoter is also required for ABA induction. In this region there is a sequence closely related to a previously known *cis*-element, *hex-3* (GACGCGTGGC). The *hex-3* sequence is a synthetic sequence that was shown to confer ABA induction when a tetramer of it was placed upstream of the −90-bp CaMV 35S promoter in transgenic tobacco (Lam and Chua, 1991). Therefore, we designated the sequence from −244 to −235 as motif III (GCCGCGTGGC). To determine whether motif I and motif III play the same role in the 40-bp ABRE, we made constructs in which motif I was replaced by motif III and vice versa. Figure 5 shows that the degree of ABA induction in Mut 5 and Mut 6 was similar and equal to that of the wild type. This result indicates that, although motif I and motif III function independently, their functions are not distinct. This suggests that motif III is not a CE like CE1, which functions as an ABRE only when associated with a motif-I-like element (Shen and Ho, 1995).

Detailed Analysis of Motif-I and Motif-III Sequences by Substitution Mutation

To determine which sequences of motif I and motif III are important for ABA induction, we introduced a series of 2-bp substitution mutations into both motifs and analyzed them by transient assays. Twenty different substitution mutations, designated M1 to M10 for motif I (Fig. 6), and GM1 to GM10 for motif III (Fig. 7), were examined. In each mutant, the corresponding 2-bp sequences of either motif I or motif III were replaced with a 2-bp unrelated DNA sequence. These mutated motifs were examined on ABA induction using transient assays. Figure 6 shows that most

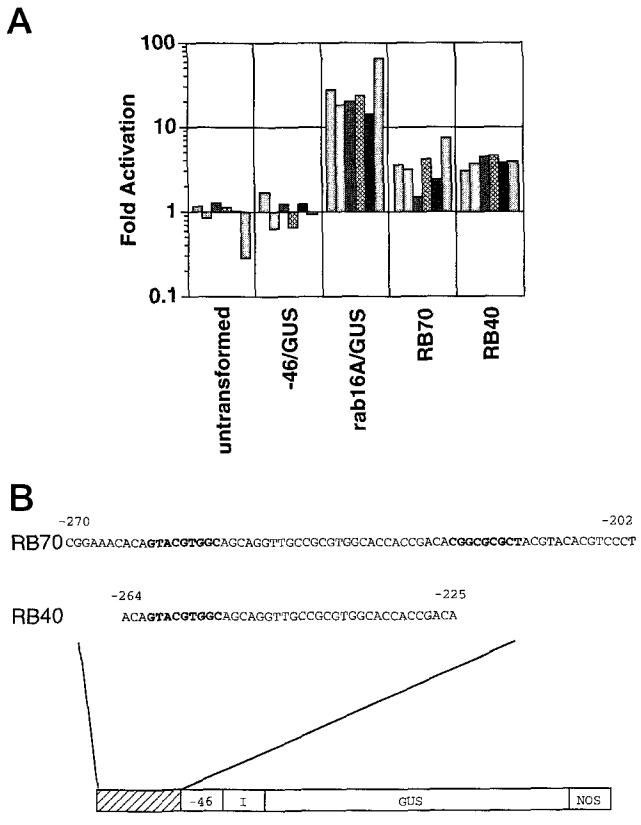

Figure 3. A, The 40-bp region of the *rab16B* promoter as an ABRE. Activation (-fold) represents the ratio of GUS activities (+ABA to −ABA) from six independent experiments. B, Schematic illustration of mutant promoters. Motif I and motif II are represented by bold letters. Mean (±SE) values of GUS activities (in pmol min^{-1} mg^{-1} protein) are: untransformed, 5.6 ± 2.5 (+ABA), 5.8 ± 1.2 (−ABA); −46/GUS, 100 ± 30 (+ABA), 94 ± 22 (−ABA); *rab16A*/GUS, 1500 ± 1500 (+ABA), 41 ± 19 (−ABA); RB70, 140 ± 60 (+ABA), 30 ± 14 (−ABA); and RB40, 570 ± 140 (+ABA), 150 ± 33 (−ABA). −46, −46-bp CaMV 35S minimal promoter; I, 190-bp castor bean catalase intron 1; GUS, *E. coli gusA*-coding region; NOS, nopaline synthase terminator.

of the mutations in motif I (those corresponding to M1-M9) resulted in complete loss of ABA induction; only M10 retained a slight ABA responsiveness. On the other hand, GM2 to GM10 in motif III showed complete loss of ABA induction, whereas GM1 still conferred ABA induction (Fig. 7). Four motif-III mutants, GM4, GM5, GM6, and GM7, showed repression of gene expression by ABA. In particular, ABA treatment clearly reduced expression of GM5 (GCCGatTGGC) to 20 to 30% of that of wild type. These results demonstrate that motif I and motif III exhibit different sequence requirements as ABREs, suggesting that they interact with different transcription factors that are involved in ABA-inducible expression.

DISCUSSION

Tissue Specificity of ABA-Inducible Gene Expression in Rice

One function of ABA is as a signal that induces expression of various genes involved in seed maturation and tolerance against stresses such as drought (Giraudat et al., 1994). ABA-inducible expression during seed maturation can be separated from that due to physiological stresses. One of the ABA-insensitive mutants of Arabidopsis, *abi3*, exhibits a seed phenotype, whereas ABA-insensitive mutants such as *abi1* and *abi2* exhibit no tissue specificity (Giraudat et al., 1994). The rice *Osem* gene, which is regulated by *Osvp1*, is expressed in the developing embryo and is not induced by ABA in vegetative tissues (Hattori et al., 1994), whereas the rice *rab16* genes are induced in vegetative tissues (Yamaguchi-Shinozaki et al., 1989). These observations suggest that there may be different pathways for

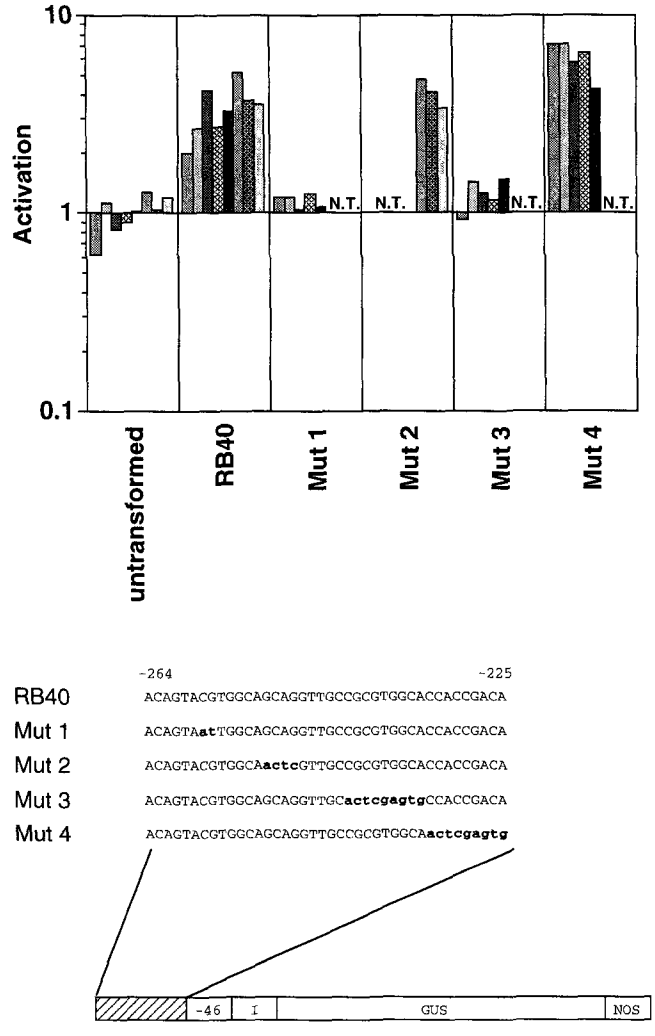

Figure 4. Substitution mutation analysis of the 40-bp ABRE. Activation (-fold) represents the ratio of GUS activities (+ABA to −ABA) from eight independent experiments. Five experiments were carried out for Mut 1, Mut 3, Mut 4, and three experiments were carried out for Mut 2. Mutations of tested plasmids are shown in lowercase bold letters. Mean (±SE) values of GUS activities (in pmol min^{-1} mg^{-1} protein) are: untransformed, 2.5 ± 0.6 (+ABA), 2.4 ± 0.3 (−ABA); RB40, 170 ± 120 (+ABA), 56 ± 45 (−ABA); Mut 1, 35 ± 19 (+ABA), 25 ± 13 (−ABA); Mut 2, 350 ± 300 (+ABA), 81 ± 58 (−ABA); Mut 3, 41 ± 22 (+ABA), 33 ± 15 (−ABA); and Mut 4, 150 ± 78 (+ABA), 23 ± 10 (−ABA). Symbols are as given in the legend to Figure 3. N.T., Not tested.

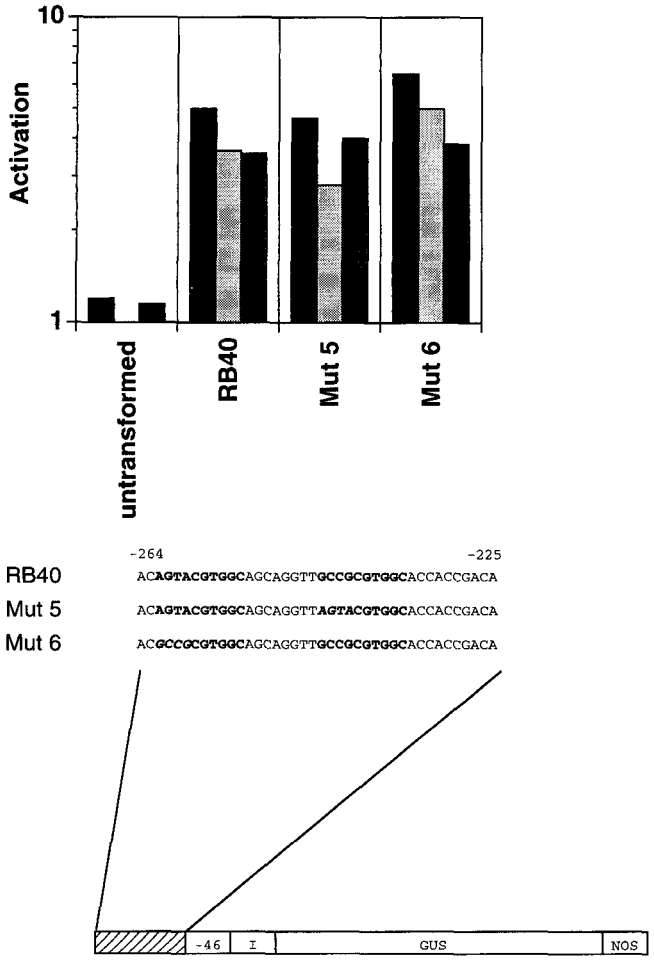

Figure 5. Motif I and motif III are interchangeable. Motif I and motif III are represented by bold letters. Mutated nucleotides are shown in italic letters. Mean (±SE) values of GUS activities (in pmol min^{-1} mg^{-1} protein) are: untransformed, 2.9 ± 0.2 (+ABA), 2.5 ± 0.1 (−ABA); RB40, 69 ± 23 (+ABA), 16 ± 4 (−ABA); Mut 5, 49 ± 14 (+ABA), 12 ± 3 (−ABA); and Mut 6, 800 ± 210 (+ABA), 150 ± 25 (−ABA). Symbols are as given in the legend to Figure 3.

ABA-inducible gene expression. In our study, transgenic rice plants showed no tissue specificity in the ABA-inducible expression of *rab16A* promoter. ABA induction of *rab16A* promoter activity was clearly observed in leaves, roots, anthers, and embryo. The low level of constitutive expression in anthers and mature embryos was detected in the absence of ABA treatment. Whether this constitutive expression of *rab16A* is due to induction by endogenous ABA or to tissue-specific expression remains to be tested. Because endogenous ABA has been shown to increase in developing embryos (Skriver and Mundy, 1990), it may be the cause of the constitutive expression of *rab16A* in embryos. No expression was detected in endosperm regardless of whether ABA was present. These observations clearly demonstrate that there is no tissue specificity in ABA induction of the *rab16A* gene. There are at least two distinct pathways for ABA-inducible gene expression in rice, considering the apparent tissue-specific ABA induction of the *Osem* gene (Hattori et al., 1995). ABA induction in roots is higher than that in leaves, suggesting that *rab16A* products play a more important role in roots than in leaves. It is surprising that *rab16A*/GUS expression in transgenic seeds is relatively low compared with those in roots. One explanation is that the *rab16A* gene product does not play an important role during embryo maturation and that other members of *rab16* gene family are important in that context. To clarify this point, the histochemical localization of expression of other *rab16* genes needs to be examined. We examined seeds derived from only one transgenic line; more independent transgenic lines should be examined in the future. Aside from ABA, both desiccation and high-salt stress induce *rab16A* gene expression. In Arabidopsis, a

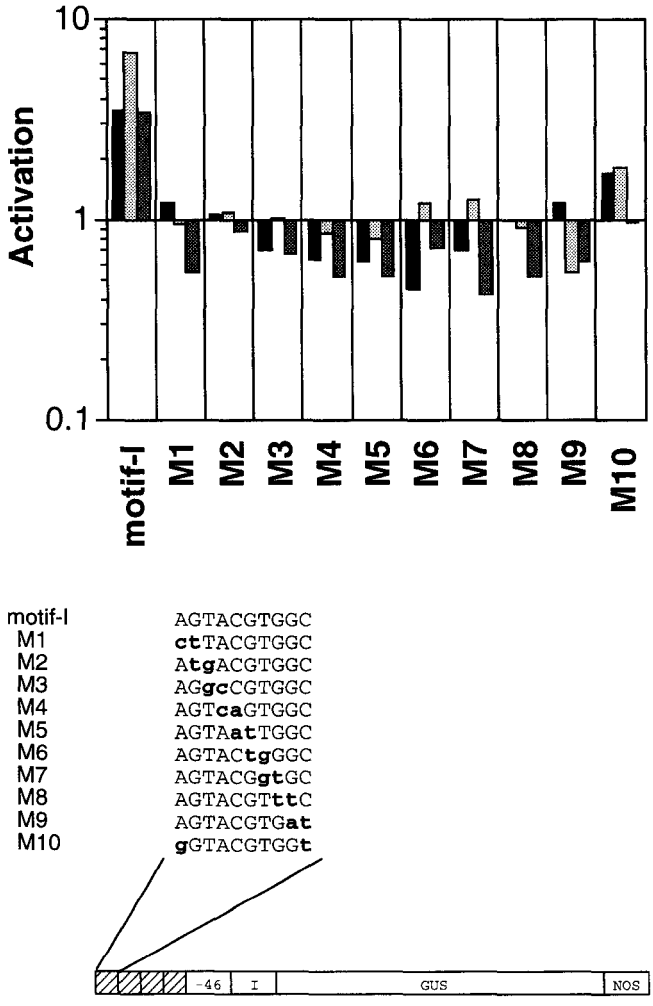

Figure 6. DNA sequence specificity of motif I on ABA response. One unit of the tetramer is shown in the bottom. Mutated nucleotides of each construct are shown in lowercase bold letters. Mean (±SE) values of GUS activities (in pmol min^{-1} mg^{-1} protein) are: motif I, 310 ± 90(+ABA) 87 ± 29 (−ABA); M1: 93 ± 33 (+ABA), 130 ± 64 (−ABA); M2, 81 ± 32 (+ABA), 85 ± 36 (−ABA); M3, 510 ± 120 (+ABA), 680 ± 190 (−ABA); M4, 15 ± 2 (+ABA), 24 ± 5 (−ABA); M5, 370 ± 110 (+ABA), 640 ± 220 (−ABA); M6, 220 ± 80 (+ABA), 310 ± 110 (−ABA); M7, 150 ± 28 (+ABA), 270 ± 110 (−ABA); M8, 320 ± 100 (+ABA), 520 ± 240 (−ABA); M9, 87 ± 30 (+ABA), 130 ± 52 (−ABA); and M10, 460 ± 95 (+ABA), 360 ± 120 (−ABA). Symbols are as given in the legend to Figure 3.

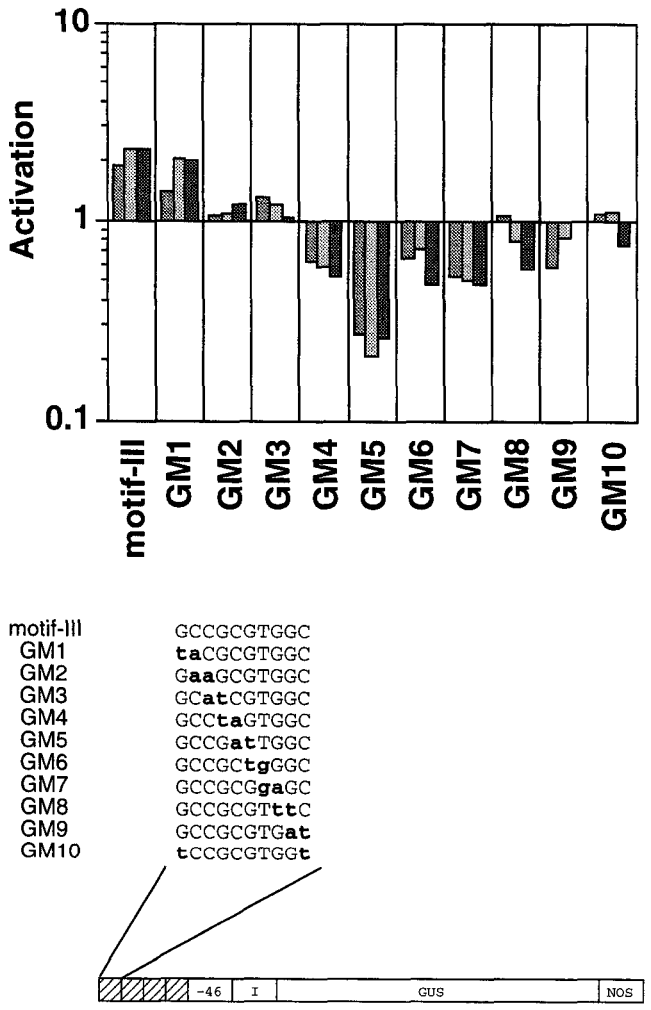

Figure 7. DNA sequence specificity of motif III on ABA response. One unit of the tetramer is shown on the bottom. Mutated nucleotides of each construct are shown in lowercase bold letters. Mean (±SE) values of GUS activities (in pmol min^{-1} mg^{-1} protein) are: motif III, 1400 ± 200 (+ABA), 580 ± 70 (−ABA); GM1, 710 ± 110 (+ABA), 380 ± 40 (−ABA); GM2, 1300 ± 200 (+ABA), 1100 ± 200 (−ABA); GM3, 140 ± 10 (+ABA), 110 ± 10 (−ABA); GM4, 80 ± 7 (+ABA), 150 ± 20 (−ABA); GM5, 27 ± 5 (+ABA), 90 ± 15 (−ABA); GM6, 85 ± 19 (+ABA), 130 ± 20 (−ABA); GM7, 340 ± 60 (+ABA), 680 ± 120 (−ABA); GM8, 470 ± 70 (+ABA), 580 ± 70 (−ABA); GM9, 1000 ± 120 (+ABA), 1300 ± 200 (−ABA); and GM10, 3000 ± 600 (+ABA), 2900 ± 500 (−ABA). Symbols are as given in the legend to Figure 3.

drought-responsive element has been identified as a *cis*-element different from ABRE in the rd29 gene (Yamaguchi-Shinozaki and Shinozaki, 1994). Future studies need to be conducted to determine whether drought induction of *rab16A* expression is separable from ABA induction.

Nature of Motif I and Motif III as ABREs

Previous studies revealed two conserved *cis*-elements termed motif I and motif II among *rab16A-D* promoters (Yamaguchi-Shinozaki et al., 1989). A hexamer of motif I conferred ABA-inducible gene expression in barley protoplasts when fused to a minimal CaMV 35S promoter, whereas motif II did not (Skriver et al., 1991). In this study, we identified a 40-bp fragment that can function as an ABRE in the *rab16B* promoter, which does not include the conserved element motif II. These results clearly indicate that motif II is not required for ABA induction. However, it is possible that motif II has other functions in vivo, because it was shown to interact with rice nuclear proteins (Mundy et al., 1991).

Scanning mutation analysis of the 40-bp fragment revealed two distinct regions of the *rab16B* promoter for ABA-inducible gene expression. One of them includes motif I (AGTACGTGGC), and the other includes GCCGCGTGGC, designated as motif III and closely related to *hex-3* (GACGCGTGGC). A tetramer of *hex-3* conferred significant ABA induction when fused to a truncated CaMV 35S promoter (−90 bp) in transgenic tobacco plants (Lam and Chua, 1991). Although the 3′ half of these two motifs is common, motif I contains an ACGT sequence that is most commonly found in other ABREs, but motif III does not. Therefore, motif I is distinct from motif III and *hex-3*. Substitution mutation analysis indicates that motif I and motif III can replace each other, so specific interaction between them is not required for ABA induction. Furthermore, mutation in either motif (Mut 1 and Mut 3 in Fig. 4) leads to complete loss of ABA induction, indicating that the effect of the mutations is not additive. Taken together, our results suggest that the presence of two tandemly aligned motifs is required for ABA induction of the *rab16B* gene: motif I/motif III, motif I/motif I, or motif III/motif III. Recently, ABRCs have been identified in the barley *HVA22* promoter (Shen and Ho, 1995), one of which is composed of a motif-I-like element, ABRE3, and a CE, CE1. Specific interaction between these two elements appears to be required for ABA regulation.

Two plant bZIP proteins, EmBP-1 and TAF-1, have been isolated as DNA-binding proteins interacting with the ABREs *Em1a* and motif I, respectively (Guiltinan et al., 1990; Oeda et al., 1991). Interaction of these binding proteins with ABREs is not modulated by ABA. Furthermore, systematic analyses of plant bZIP proteins revealed that they exhibit a relaxed binding specificity for DNA sequence motifs containing an ACGT core (Izawa et al., 1993). Therefore, it remains to be understood which bZIP proteins or other transcription factors are involved in ABA induction in vivo. The ACGT sequence apparently functions as a core sequence when plant bZIP proteins interact with DNA. When one of the strongest binding sequences of plant bZIP proteins, GCCACGTGGC, was mutated to GCCGCGTGGC, no bZIP protein could interact with the mutated sequence (Izawa et al., 1993). Because this mutated sequence is the same with the motif-III sequence, it is unlikely that motif III interacts with ACGT-recognizing bZIP proteins in vivo. On the other hand, motif I is an ACGT element that significantly interacts with several bZIP proteins in vitro (Oeda et al., 1991; Izawa et al., 1993), suggesting an interaction between motif I and bZIP proteins in vivo. It was previously shown that a synthetic heterodimer between bZIP proteins that recognize either

ACGT or GCGC interacts with a GCGT sequence as a core sequence (Cao et al., 1991; Vinson et al., 1993). Therefore, it is not impossible that motif III can interact with such a heterodimer in vivo.

DNA Sequence Specificity of ABREs

The requirement of two distinct *cis*-acting elements to mediate specific gene expression by various signals is often found in plant genes. In the parsley *chs* gene box I GTCCCTCCAACCTAACC and the ACGT element called the G box are both required for UV induction (Schulze-Lefert et al., 1989). Likewise, the G box is thought to interact with box II (GGTTAA) and I-box (GATAAG) motifs in coordinating light-regulating expression of the *rbcS*-A gene (Donald and Cashmore, 1990). Furthermore, the G box in the bean *chs15* gene needs an H box (CCTACC-N_7-CT) to respond to 4-CA (Loake et al., 1992). In the barley *Amy32b* gene, a CE (O2S) was shown to allow a single copy of either a GA-responsive element or an ABRE to mediate the hormonal effects in barley endosperm (Rogers and Rogers, 1992). Recently, ABRC1 of the barley *HVA22* promoter was shown to comprise ABRE 3 and CE1 (TGCCACCGG) (Shen and Ho, 1995). These results clearly indicate that combinations of two distinct *cis*-elements are required for specific gene expression. However, both the hexamer of motif I and the tetramer of *hex-3* have been shown to function as ABREs in barley protoplasts and transgenic tobacco plants, respectively (Lam and Chua, 1991; Skriver et al., 1991). These results indicate that either motif I or *hex-3* alone can be an essential component of the ABRE. The present study shows that each tetramer of motif I and motif III is essential for ABA induction in rice protoplasts and that two tandemly aligned motifs are required for ABA induction (Fig. 5). Considering the sequence specificity of the motifs as ABREs (Figs. 6 and 7), these two motifs might interact with different transcription factors. Therefore, a protein factor able to interact with both motif I- and motif III-binding proteins might be involved in ABA-inducible gene expression.

All mutated motif I in Figure 6 reduced or lost ABA inducibility. According to plant bZIP-binding specificity analysis (Izawa et al., 1993), only the M9 mutation is likely to interact with plant bZIP proteins. The M9 mutation may exhibit weaker affinity than motif I for bZIP proteins. Taken together, mutations in motif I that likely cause a loss or reduction in the affinity with bZIP proteins show a loss of ABA-inducible expression, suggesting that motif I may interact with bZIP proteins in vivo. On the other hand, it is unlikely that motif III interacts with ACGT-recognizing bZIP proteins (Izawa et al., 1993). The GM1 mutation in motif III did not alter ABA-inducible expression, whereas other mutations caused lost or reduced ABA-inducible expression. Therefore, this study reveals that a tetramer of GM1, TACGCGTGGC, can function as an ABRE in rice protoplasts, suggesting that the 5′ site of motif III may exhibit relaxed sequence specificity as an ABRE. Furthermore, GM5 clearly exhibits suppression by ABA, whereas the same mutation (CG to AT) in the corresponding region of motif I (M5) does not. How GM5 mutation can alter the response from activation to suppression by ABA remains to be discovered.

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