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Nuclear proteins bind conserved elements in the abscisic acid-responsive promoter of a rice *rab* gene

(cis-acting elements/DNAse 1 cleavage inhibitor pattern/osmotic stress/PEG-mediated transfection/trans-acting factors)

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ABSTRACT **We have previously shown that the expression of a rice gene, *rab-16A*, is responsive to abscisic acid (ABA) and osmotic stress in plant tissues and cultured suspension cells. We demonstrate here that transcriptional elements between −294 and −52 of this gene are sufficient to confer ABA-dependent expression on the chloramphenicol acetyltransferase reporter gene in rice protoplasts. Sequence motifs within this 242-base-pair region of the *rab-16A* gene are conserved among the 5′ upstream regions of other ABA-responsive genes. Gel retardation and DNAse I experiments show nuclear factor(s) binding to these sequences. This correlative data indicate that these motifs are involved in the transcription of the *rab* genes and suggest that they may be ABA-responsive-elements (*ABRE*s).**

The hormone abscisic acid (ABA) appears to mediate physiological processes in response to osmotic stress. Levels of endogenous ABA increase in tissues subjected to osmotic stress because of high osmoticum, salt, desiccation, or cold (1–3). Under these conditions, specific genes are expressed that can also be induced in unstressed tissues by the application of exogenous ABA (4–6). A number of these ABA-responsive genes are normally expressed during late embryogenesis, when seed tissues desiccate and the embryos of some species become dormant (7–9). Therefore, it is thought that some of these ABA-responsive genes encode proteins with osmoregulatory or other protective functions (10–14).

We are interested in elucidating how ABA regulates specific gene expression because of its role in seed development and in the response of plants to osmotic stress, two agronomically important traits. To this end, we have characterized an ABA-responsive rice gene, initially called *rab-21*, that is expressed in seeds late during embryogenesis and that is induced by ABA and osmotic stress in vegetative tissues (6). We now have completely characterized a rice locus encoding this gene and its three tightly linked homologues (15). In the present and subsequent publications we call these genes *rab-16A–D*, in keeping with the average molecular weights (16,000) of the encoded RAB proteins.

Comparison of the 5′ upstream sequences of *rab-16A–D* and other ABA-responsive genes reveals two conserved motifs that could be involved in ABA responsiveness. To identify such cis-acting ABA responsive elements (*ABRE*s), we prepared a series of 5′ deletion mutants and chimeric promoter constructs to assay their activities in protoplasts prepared from rice suspension cultures. Using these constructs, we present evidence here to show that the *rab-16A* gene is transcriptionally regulated by ABA. Furthermore, we provide *in vitro* data indicating that the conserved sequence motifs in the *rab-16A* promoter specifically bind nuclear protein factors. Therefore, these motifs may be candidate *ABRE*s.

METHODS

DNA Manipulations. All DNA manipulations were performed by standard procedures (16). Deletions of the 5′ upstream region (*Xba* I/*Nhe* I; positions −2500 to +27) of the *rab-16A* gene (6), cloned in pEMBL 12+ (17), were generated by using BAL-31 exonuclease digestion. Plasmid DNAs used in all experiments were purified by CsCl gradient centrifugation, chromatographed on Sephadex G-50F in TE buffer (10 mM Tris/1 mM EDTA, pH 8.0) (0.4 × 40 cm), and collected by ethanol precipitation.

Reporter Gene Constructs. Three types of promoter constructs were fused to the bacterial chloramphenicol acetyltransferase (CAT) coding region with a pea *rbcS-E9* polyadenylylation site (18). The three types of plasmid construct are: (*i*) a long 35S promoter (produces a 35S transcript) from position −941 to +8; (*ii*) *rab-16A*/35S chimeric promoters containing *rab-16A* 5′ deletion fragments (starting at positions −1505, −770, and −442) that are truncated at −52 (*Sac* I site) and fused to the 35S promoter TATA box (from −90 to +8); and (*iii*) *rab-16A* 5′ deletion fragments (starting at −442, −294, and −52) fused to the CAT-encoding region at +27 of the *rab-16A* promoter.

Transient Expression Assay in Rice Protoplasts. Rice suspension cells were cultured from embryo-derived callus of Tapei 309 in standard media (19). PEG-mediated transfection was used to introduce the constructs into rice protoplasts as described by Krens *et al.* (20). Twenty-four hours after transfection, half of the protoplasts were incubated in 10 μM ABA, harvested 18 hr later, and assayed for CAT activity as described by Nagy *et al.* (21).

Preparation of Binding Protein Extracts. Whole-cell protein extracts were prepared from roots and shoots of 20-day-old rice seedlings grown hydroponically as described by Green *et al.* (22). Nuclear extracts were regularly prepared from shoots of 9-day-old dark-grown plants (22). For ABA treatments, leaves were sprayed with 100 μM ABA/0.02% Tween at 24 hr, 12 hr, and 3 hr before harvest. The average yield of protein per nuclear extract was 100 mg per kg of leaves (3.5 liters of seeds as starting material).

Gel Retardation and DNAse I Cleavage Inhibition Patterns (Footprinting). Probes were labeled at the polylinker *Hin*dIII site (5′ end of *rab-16A*; fragment from −290 to +27) with Klenow enzyme, digested with *Sac* I (cleavage at −52, for gel retardation assays), isolated on 4% polyacrylamide gels, and characterized by isotachophoresis. Competitor fragments were

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Abbreviations: ABA, abscisic acid; *ABRE*, abscisic acid-responsive DNA element; *rab*, gene responsive to abscisic acid.

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isolated on agarose gels by using DE-81 nylon membranes (Schleicher & Schuell NA-45). A typical binding reaction contained 20 fmol of probe (5,000–20,000 cpm) and a 100-fold molar excess of specific inhibitor DNA. Gel retardation assays were performed as described by Green *et al.* (22) with nuclear extract protein at 0.6 $\mu g/\mu l$, poly(dI)·poly(dC) at 0.5 $\mu g/\mu l$, and poly(dA)·poly(dT) at 2 $\mu g/\mu l$ as competitors. DNAse I footprint assays were performed with extract protein at 4 $\mu g/\mu l$, poly(dI)·poly(dC) at 1 $\mu g/\mu l$, and poly(dA)·poly(dT) at 5 $\mu g/\mu l$ as competitors (22).

RESULTS

5′ Upstream Sequences of the *rab-16A* Gene Confer Responsiveness to ABA upon the CAT Reporter Gene. We showed previously that accumulation of rab-16 transcripts is inducible in various rice tissues by ABA and osmotic stress (6). To examine whether this induction is due at least in part to transcriptional control by *rab-16* 5′ upstream sequences, we constructed gene fusions using 5′ upstream fragments of the *rab-16A* gene and the bacterial CAT reporter gene. These gene constructs were introduced into rice protoplasts by using PEG-mediated transfection to assay for transient, ABA-responsive CAT enzyme expression. Fig. 1*A* outlines the gene constructs used, and Fig. 1*B* shows the results of a typical CAT enzyme assay. The control transfections using constructs 1 and 2 clearly show that CAT expression is promoter dependent and that transcription from the strong 35S viral promoter is not responsive to ABA.

Transfections with the chimeric *rab-16A*/35S constructs 3–5 (*rab* deletion fragments starting at −1505, −770, and −442, respectively) show low levels of CAT activity in protoplasts incubated without ABA. This low-level, constitutive expression may be due to cumulative effects of promoter elements within the *rab-16A* gene sequences upstream of −52 and elements within the 35S promoter fragment from −90 to +8. This region of the 35S promoter, which contains the putative TATA box, has recently been shown to contain elements capable of enhancing transcription in tobacco cells (18). Therefore, it is not surprising that it affects basal level expression in our system.

Of greater significance is the fact that such chimeric promoters are responsive to ABA. This can be clearly seen in the transfection experiments with the shorter promoter containing the *rab-16A* 5′ upstream fragment from position −442 to −52 (construct 5), as well as that with the fragment from −770 to −52 (construct 4). A comparison of the three chimeric promoters assayed (constructs 3–5) suggests that silencer-like sequences that appear to diminish ABA responsiveness reside upstream of −440 in the *rab-16A* DNA. Similar reductions in ABA-inducible promoter strength with increasing promoter length have been seen in comparable assays of 5′ upstream sequences of the wheat *EM* gene with its own TATAA box (23). Therefore, it is likely that this "silencing" effect is independent of the presence of 35S sequences in our chimeric constructs.

Transfections with constructs 6 and 7 show that sequences between −290 and +27 of the *rab-16A* gene confer strong, ABA-dependent expression on the CAT reporter gene. CAT enzyme activity in control (−ABA) incubated protoplasts was undetectable, while incubation with 10 μM ABA resulted in CAT levels comparable to that seen in protoplasts transfected with constructs containing the full 35S viral promoter. Protoplasts transfected with construct 8 do not express detectable levels of CAT, indicating that sequences between −52 and +27 do not mediate the ABA responsiveness of the *rab-16A* gene. This is further supported by the fact that constructs 4 and 5, which lack this region, are clearly responsive to ABA. Therefore, we conclude that sequences between −290 and −52 of the *rab-16A* gene contain ABA-responsive DNA elements that modulate the transcription of this gene.

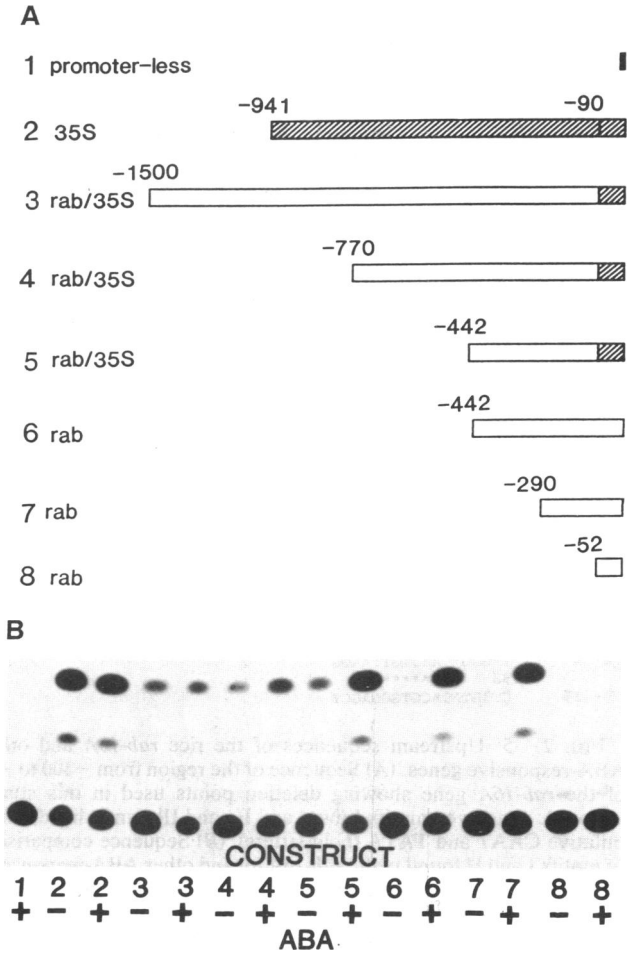

FIG. 1. Transient expression of CAT gene fusions in rice protoplasts. (*A*) CAT fusion constructs with the 5′ upstream sequences of the cauliflower mosaic virus 35S transcription unit and *rab-16A* gene used for transfection. The constructs are: 1, negative control, "promoterless" CAT gene; 2, positive control, constitutive 35S promoter (−941 to +8); 3, chimeric *rab-16A*/35S promoter [*rab-16A* fragment from −1505 to −52 fused to 35S promoter TATA box (−90 to +8)]; 4, *rab-16A* fragment from −770 to −52 fused to 35S promoter TATA box; 5, *rab-16A* fragment from −442 to −52 fused to 35S promoter TATA box; 6, *rab-16A* fragment from −442 to +27 of *rab-16A* promoter, fused to CAT gene (−442/+27 *rab-16A*/CAT); 7, −294/+27 *rab-16A*/CAT; 8, −52/+27 *rab-16A*/CAT. (*B*) CAT assays of rice protoplasts transfected with the above constructs and incubated without or with 10 μM ABA as marked.

Sequence Motifs Between −294 and −52 of the *rab-16A* Gene Are Conserved Among 5′ Upstream Regions of Other ABA-Responsive Genes. Fig. 2*A* shows the sequence of the proximal 300 base pairs (bp) of the *rab-16A* promoter (6). Our transfection experiments indicate that sequence elements within this region are capable of controlling ABA-dependent gene expression. Such *ABRE*s might be expected to be conserved within the 5′ upstream sequences of the *rab-16A* and other ABA-responsive genes. We have previously noted that several G+C-rich sequence motifs are duplicated within the *rab-16A* promoter. More recently, we have sequenced three other *rab-16* genes (*rab-16B–D*; ref 15). These genes contain single copies of one of the G+C-rich motifs noted in the *rab-16A* gene (Fig. 2*B*, motifs IIa and IIb). Furthermore, the upstream regions of all of the rice *rab-16* genes contain a motif that is also found in the upstream regions of ABA-responsive genes from cotton (Fig. 2*B*, motif I).

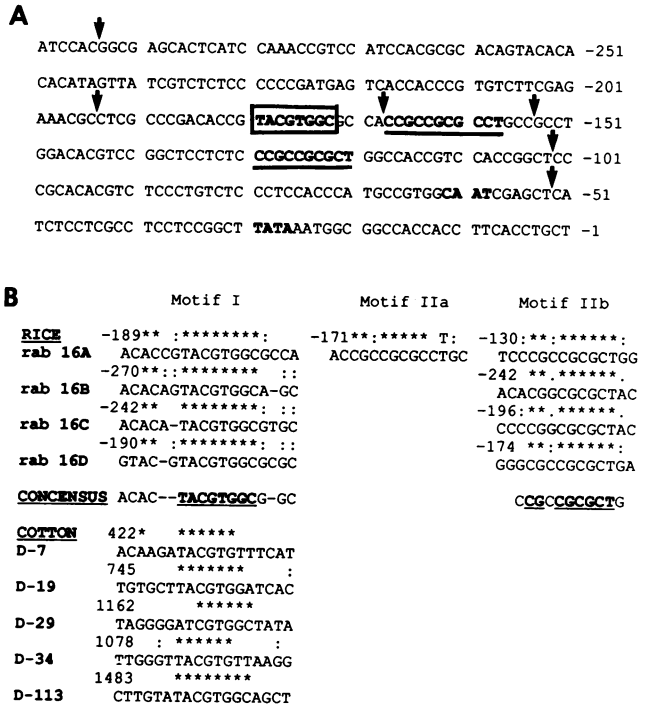

FIG. 2. 5′ Upstream sequences of the rice *rab-16A* and other ABA-responsive genes. (*A*) Sequence of the region from −300 to −1 of the *rab-16A* gene showing deletion points used in this study (arrows), conserved motifs I (box) and IIa and IIb (underlined), and putative CAAT and TATA (bold letters). (*B*) Sequence comparison of motifs I and II found in the *rab-16A* (6) and other ABA-responsive genes. *rab-16B–D* are the three other tightly linked members of the *rab-16* locus of rice (15). *Lea-D7–113* are cotton genes described by Baker *et al.* (8). ★, Base conserved within the motif among all four *rab-16* genes; :, base conserved within the motif among three *rab-16* genes.

Examination of the motif I sequence shows that it has some homology to the cyclic AMP-responsive element described by Deutsch *et al.* (24). We have noted (25) that motif II is similar to the binding site of SP1, a mammalian transcription factor. Visual and computer analysis did not identify any regions of significant homology between *rab-16A* upstream sequences and steriod hormone-responsive elements identified in mammalian genes (26).

The Conserved Sequence Motifs I, IIa, and IIb Are Sites for Nuclear Protein Binding. To attempt a finer delination of the regulatory regions of the *rab-16A* promoter, we examined whether sequences within the region from −290 to +27 specifically bind cellular or nuclear protein factors. In initial experiments, whole-cell protein extracts were prepared from control and ABA-treated roots or shoots. We have shown previously that both of these tissues accumulate rab-16A mRNA in response to ABA. No discrete binding was seen between proteins in these extracts and the *rab-16A* probe. However, discrete binding was achieved in experiments with proteins extracted from shoot nuclei (Fig. 3). Competition experiments with unlabeled upstream fragments (Fig. 3*A*) suggest that two different binding sites occur between −194 and −102, one of them lying upstream of −154 (Fig. 3*B*, see arrows). The simplest explanation for these gel retardation results is that the slower complex is due to factor(s) binding to the type II motif, while the faster one is a complex with motif I.

This pattern of binding was consistently seen for proteins extracted from shoot nuclei of both control and ABA-treated plants (data not shown). Although the level of this binding (cpm/μg of protein) in extracts from ABA-treated shoots was

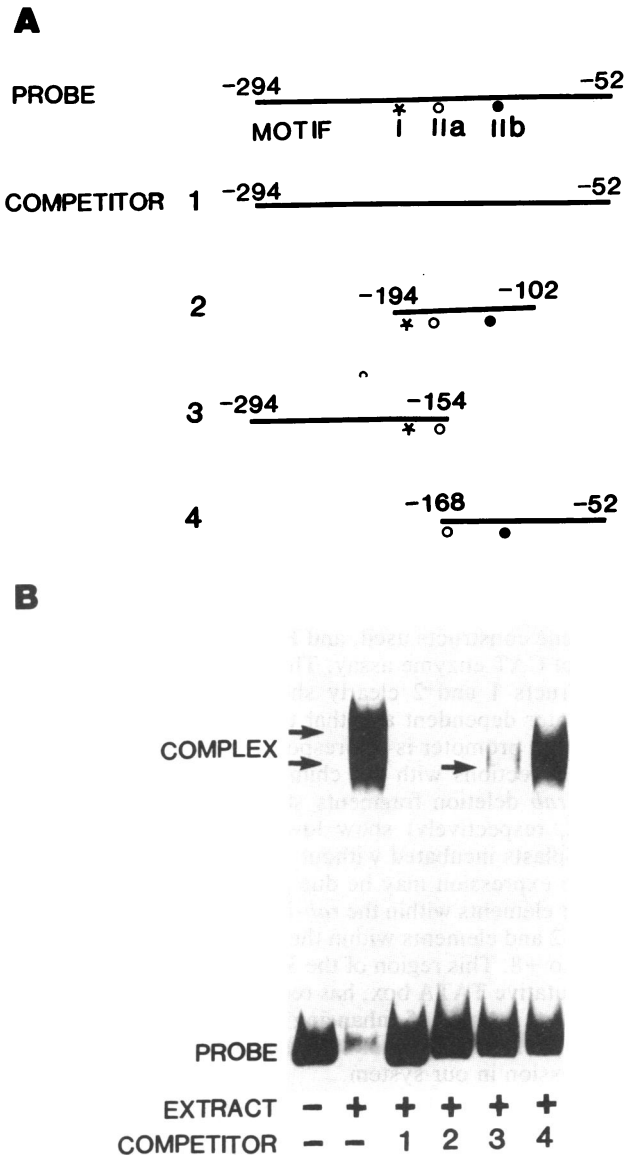

FIG. 3. Gel retardation assay delineating a region of the *rab-16A* promoter that binds nuclear factor(s). (*A*) *rab-16A* 5′ upstream fragments, generated by BAL-31 exonuclease digestion, used as probes and as the following competitors: 1, cold probe fragment from −294 to −52; 2, region containing motifs I, IIa, and IIb, from −194 to −101; 3, region containing motif I and motif IIa, from −294 to −154; 4, region containing motif IIb, from −168 to −52. Motifs I (★), IIA (○), and IIB (●) occur as marked. See *Methods* for probe/competitor concentrations. (*B*) Gel retardation experiment assessing binding of nuclear proteins from ABA-treated leaves to the *rab-16A* promoter region from −294 to −52.

sometimes 2- to 3-fold higher than in extracts from control shoots, the data suggested that binding of these factors *in vitro* is not significantly increased by ABA. Addition of ABA to the binding reactions also had no effect on the levels of specific DNA–protein complexes formed.

To further define the sequence(s) that interact with nuclear proteins, we carried out DNAse I footprinting experiments. Fig. 4 shows what appears to be two major areas of protection (positions −105 to −131 and −155 to −180) that coincide with the conserved sequence motifs I and IIa/IIb. The presence of two bands in the gel retardation assays (Fig. 3) and the bipartite nature of the footprint suggest that binding of a factor to motif I may be independent of binding to motifs IIa or IIb. Taken together with the *in vivo* activities of the 5′

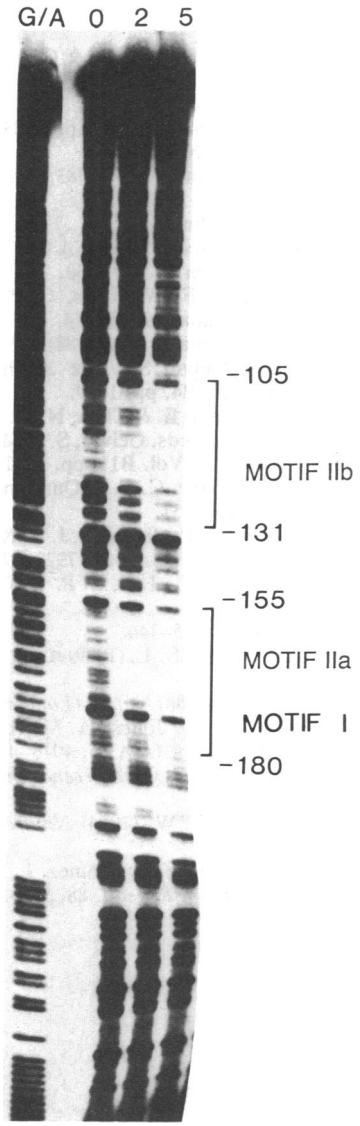

FIG. 4. DNAse I footprint experiment showing the binding sites of nuclear protein(s) in the region from −290 to +27 of the *rab-16A* promoter. The probe is the 3′ end-labeled bottom strand of the deleted promoter region shown in Fig. 2*A*. Lane G/A shows the G+A product ladder derived by chemically sequencing the probe. The amount ($\mu g/\mu l$) of nuclear extract used to generate the footprint is given above the remaining lanes.

deletion mutants, these *in vitro* results indicate that motifs I and II may be important in some aspect of the transcription of *rab* genes, most probably their response to ABA.

DISCUSSION

We began our study of the molecular mechanism of plant hormone action by isolating the members of a rice gene family, the *rab-16A–D* genes, whose expression is strongly induced by ABA (6, 15). We are analyzing the ABA-responsive expression of the *rab-16* genes as a model system to elucidate the mechanism of action of this hormone. In the present work, we show that the region between positions −294 and +27 in the 5′ upstream region of the *rab-16A* gene is sufficient to confer ABA-responsive, transient expression upon the CAT reporter gene in transfected rice protoplasts. These results are similar to those reported by Marcotte *et al.* (23), who showed that sequences between −550 and +95 of the wheat *Em* gene confer ABA induction upon the *GUS* reporter gene in rice protoplasts. Our experiments also indicate that the *rab-16A* region between positions −290 and −52 alone acts to enhance the expression of the CAT gene when placed 5′ to a heterologous TATA box from the constitutively expressed 35S cauliflower mosaic virus gene. These results indicate that this 242-bp region of the *rab-16A* gene contains one or more *ABRE*s.

Comparison of the 5′ upstream sequences of the *rab-16A–D* genes and those from several ABA responsive, late embryogenesis abundant (*lea*) genes from cotton (8) revealed conserved sequence motifs that are good candidates for *ABRE*s. Two motifs were found to be conserved in all four *rab-16* genes. Motif I has the consensus RTACGTGGR (R is an unspecified purine nucleoside), which is similar to the cAMP-responsive element (TGACGTCA) that binds the transcription factor *CREB* (24). More importantly, motif I is found in the 5′ upstream regions of five of six *lea* genes and in that of the ABA-responsive wheat *Em* gene (R. Quatrano, personal communication). Motif II, which is found in two copies (IIa and IIb) in *rab-16A* and once in *rab-16B–D* , has the consensus CGSCGCGCT, in which S is G or C. It occurs in the *rab-16* genes as part of sequences that are similar to the degenerate decanucleotide binding site of *SP1*, an auxilliary mammalian transcription factor (25).

The foregoing results prompted us to test whether the region of the *rab-16A* gene from −290 to −52, and in particular the conserved motifs I and II, specifically bind cytosolic or nuclear proteins. Gel retardation studies showed that this upstream region indeed binds nuclear protein(s), forming what appears to be two discrete DNA–protein binding complexes limited to the region containing motifs I, IIa, and IIb (from −192 to −102). At this level of resolution, it is not possible to determine whether a single protein or two protein species bind to this region. These complexes are formed at only slightly higher levels by nuclear proteins extracted from ABA-treated tissues than by those from control tissues. This suggests that binding of the factor(s) is not promoted by the hormone *in vitro*. This "constitutive" binding has been noted for various activator and regulatory factors (26, 27). Such factors apparently activate transcription only when modified by interaction with other proteins or by changes in their phosphorylation state.

DNAse I footprinting enabled us to show that the detectable sites of nuclear protein binding to the *rab-16A* promoter are limited to the sequences containing the conserved motifs I, IIa, and IIb. These correlative data indicate that these sequences are involved in the transcription of the *rab-16* genes, and possibly of the *lea* genes, and suggest that motifs I and II are candidate *ABRE*s.

Work on animal hormones has defined two major pathways of hormone action: (*i*) activation of regulatory factors by direct steroid hormone binding (26) and (*ii*) activation via "second messenger" pathways (24). Recent studies show that the response of cells to auxin involves plasmalemma receptors and phosphatidylinositol metabolites, suggesting that second messenger pathways mediate the action of this hormone (28, 29). The results presented here do not permit us to discern whether these mechanisms mediate ABA-responsive expression of the *rab-16* genes. Evidence from other tissue systems suggests that plasmalemma ion-transport proteins (30), receptors (31), and protein phosphorylation (32) are involved in cellular responses to ABA. However, we were unable to detect changes in *rab-16* gene expression in cultured rice suspension cells incubated with bromo-cAMP, phorbol 12-myristate 13-acetate forskolin, Ca^{2+}, or a Ca^{2+} ionophore—molecules that affect second-messenger signaling in animal cells (unpublished data). We hope to use defined *ABRE*s as probes for identifying clones encoding factors binding to the *rab-16A* promoter. The characterization of these factors will provide useful tools with

which to dissect the pathway(s) of ABA-induced gene expression.

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