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Abscisic Acid-Responsive Sequences from the Em Gene of Wheat

William R. Marcotte, Jr.,[1] Sandra H. Russell, and Ralph S. Quatrano[1,2]

Molecular Biology, Central Research and Development, Experimental Station, E.I. du Pont de Nemours and Co., Inc., Wilmington, Delaware 19880-0402

We demonstrate that a chimeric gene containing the β-glucuronidase (GUS) reporter gene linked to a 646-base pair 5′ fragment (−554 to +92) from the abscisic acid (ABA)-regulated Em gene from wheat is correctly expressed in transgenic tobacco. We observe high activity only in embryos of mature seeds, and immature seeds cultured on ABA show enhanced expression. Using a rice transient assay, we identify a 260-base pair fragment (−168 to +92) that accounts for the ABA-specific 15-fold to 20-fold increase in GUS expression. A 50-base pair sequence (−152 to −103) fused 5′ in either orientation to a truncated cauliflower mosaic virus promoter (35S) increases GUS activity threefold in the presence of ABA. Insertion of the Em 5′-untranslated region (+6 to +86) between the 35S promoter and the ATG of GUS results in a 10-fold increase in GUS activity in the absence of ABA. These results suggest the following two functional fragments of the Em 5′ region: an ABA response element from −152 to −103 and an element between +6 and +86 that quantitatively increases the ABA response.

INTRODUCTION

The molecular basis of phytohormone action has been a subject of intense interest to plant physiologists for decades. Within the last several years, molecular techniques have been used to demonstrate that certain phytohormones, most notably gibberellic acid (GA) (Chandler et al., 1984; Jacobsen and Beach, 1985), auxin (Theologis, Huynh, and Davis, 1985; Ainley et al., 1988; McClure et al., 1989), ethylene (Broglie et al., 1989), and abscisic acid (ABA) (Gomez et al., 1988; Mundy and Chua, 1988; Williamson and Quatrano, 1988), modulate mRNA levels of specific genes in different tissues from a wide variety of plants. In most cases, a component of the regulation by hormones is at the level of transcription, but post-transcriptional controls may also be operative (e.g., Williamson and Quatrano, 1988).

DNA sequences regulating genes responsive to signals other than hormones, such as light, heat, wounding, and anaerobic stress, have been characterized in some detail (for review, see Benfey and Chua, 1989). Whereas genomic clones have been isolated and sequenced for a number of hormonally activated genes (e.g., Huttly, Martienssen, and Baulcombe, 1988; Mundy and Chua, 1988; McClure et al., 1989), and sequences in the 5′ upstream regions of genes similarly regulated by GA, ethylene, or auxin show distinct homologies (Huttly, Martienssen, and Baulcombe, 1988; Broglie et al., 1989; McClure et al., 1989), a functional analysis is required to identify sequences in these regions that are responsible for mediating a hormonal response.

Because many monocots cannot be reproducibly used for transgenic analysis, we developed a transient expression assay to identify regions of ABA-responsive genes that mediate the hormonal response (Marcotte, Bayley, and Quatrano, 1988). We demonstrated the expression and normal regulation of an ABA-inducible promoter from wheat (Em) in rice protoplasts using a 646-bp segment from the Em gene (−554 to +92) linked to the reporter gene β-glucuronidase (GUS) and the cauliflower mosaic virus (CaMV) 3′ end. After introduction of this construct into the rice protoplasts via polyethylene glycol-mediated DNA uptake, a consistent 15-fold to 30-fold increase in GUS activity was observed upon the addition of ABA. This induction was within hours of ABA addition and was proportional to the concentration of ABA. RNase protection experiments showed that the transcription start site in the rice protoplasts (86 bp from the ATG) was the same as that in ABA-treated wheat embryos. Furthermore, protected fragments were detected only in RNA isolated from ABA-treated samples, suggesting that transcriptional controls were involved in ABA regulation (Marcotte, Bayley, and Quatrano, 1988).

A recent report by Huttly and Baulcombe (1989) demonstrated that a GA-responsive α-amylase promoter from wheat (*α-Amy2/54*) responds after several days of exposure to exogenous GA in an oat aleurone protoplast transient assay. Sequences within 300 bp of the transcription

[1] Current address: Department of Biology, University of North Carolina, Chapel Hill, NC 27599-3280.

[2] To whom correspondence should be addressed.

start of *α-Amy2/54* were sufficient to direct a specific, high level of expression in this transient assay. In addition, Broglie et al. (1989) expressed an ethylene-inducible bean chitinase gene in transgenic tobacco, and demonstrated by deletion analysis that a segment of approximately 200 bp contains sequences necessary for ethylene-regulated expression. Hence, functional analyses using transient systems and transgenic plants have identified regions several hundred base pairs from the transcription start of hormonally regulated genes that appear to contain sequences necessary for the specific induction of genes by ethylene, GA, or ABA.

What are the sequences in the Em gene of wheat that mediate the ABA response? Toward this end, we demonstrate in this paper that a 5′ region of the wheat Em gene that responds to ABA in the rice transient assay can also direct the expression of GUS in tobacco. This expression is temporally and spatially identical to the expression of the Em gene in wheat (i.e., in mature embryos), and can be precociously induced with exogenous ABA. This confirms, and extends to hormonally regulated genes, several previous studies indicating that results from analyses of 5′ regulatory sequences in transient assays are similar to those from transgenic experiments (e.g., Ellis et al., 1987; Walker et al., 1987) and that some promoters from monocots can function normally in dicots (see Benfey and Chua, 1989; Robert, Thompson, and Flavell, 1989). Furthermore, using the rice transient system in this study, we show that a 260-bp fragment (−168 to +92) can account for the full ABA-specific 15-fold to 20-fold increase in GUS expression. Sequence comparisons with other ABA-regulated genes show conserved sequences within this region. Using this information, we dissected this fragment further and show that a 50-bp oligonucleotide corresponding to −152 to −103 of the Em 5′ region can, in either orientation, confer ABA responsiveness to a truncated CaMV promoter. The response using the oligonucleotide was, however, fivefold to 10-fold lower than the response using the full 260-bp segment of the Em 5′ segment. A fragment in the 5′-untranslated region of Em (+6 to +86), although not responsive to ABA specifically, is shown to enhance GUS expression from the truncated CaMV 35S promoter by 10-fold in the absence of ABA. These results suggest two functional fragments of the Em 5′ region: an ABA response element from −152 to −103 and an element between +6 to +86 that may quantitatively increase the ABA response.

RESULTS

Analysis of Transgenic Plants

To test whether a 646-bp fragment (−554 to +92) of the Em gene from wheat can be appropriately regulated in tobacco, we made binary constructs containing the following chimeric genes: the 646-bp Em fragment 5′ of GUS [pBM1130 (forward) and pBM3110 (reverse orientation)] and the 35S promoter 5′ of GUS (pBM1170). These were then transformed into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating, and tobacco leaf discs were transformed as described (see Methods). Hybridization analysis using selected probes demonstrated the presence of the appropriate construct in transgenic plants (data not shown).

Primary transformants were assayed for GUS activity in various tissues. Transformants containing the pBM1130 or pBM3110 constructs exhibited no detectable GUS activity in vegetative tissue. Seeds from the transformed plants were also analyzed at several stages of development: prior to 12 days after anthesis (daa), 12 daa to 16 daa, and 16 daa to 20 daa. Table 1 shows that seeds from the pBM1130 transformants had very low GUS activity prior to 16 daa but accumulated GUS to extremely high levels by 20 daa, before complete desiccation. Seeds younger than 12 daa from the pBM1170 transformants showed GUS activity, and this activity declined during the next 8 days. The pBM3110 transformants showed no detectable GUS activity in seeds, or any other part of the plant tested, at any stage of development (data not shown).

Previous work demonstrated that Em mRNA and protein

Table 1. β-Glucuronidase Activity in Seeds from Transgenic Tobacco

Construction	Seed Age[a]	Activity[b]
	daa	
pBM1170	<12	1,037
pBM1170	12–16	307
pBM1170	16–20	269
pBM1130	<12	2
pBM1130	12–16	8
pBM1130	16–20	29,000

Seeds at different stages were removed from individual capsules of a single transformant and stored frozen at −70°C. Samples were homogenized in Eppendorf tubes using disposable pestles (Costar), and extracts were assayed for GUS activity. Seeds from nontransformed plants showed no GUS activity. More than nine individual transformants were assayed for each construction, with approximately 75% showing the pattern above.

[a] Tobacco seed and embryo development were staged according to Chen, Pan, and Beachy (1988). Earlier than 12 daa, the seeds were white, with embryos <0.2 mm; between 12 daa and 16 daa, the seeds were tan, with embryos about 0.6 mm; and between 16 daa and 20 daa, the seeds were brown (not dry), with the embryos about 0.8 mm.

[b] Activity is in picomoles of 4-methylumbelliferone per microgram of protein per minute for the Em-GUS (pBM1130) and 35S-GUS (pBM1170) constructs.

accumulate only in embryos of developing wheat seeds, reaching maximum levels late in maturation (Williamson, Quatrano, and Cuming, 1985). To determine the localization of GUS activity in seeds of transformed tobacco, seeds from the pBM1130 transformants were stained with the histochemical dye for GUS activity, as described by Jefferson (1987), and examined by microscope. Figure 1 shows that staining was confined to the embryo of the seed, the same tissue specificity seen for Em expression in wheat grains. Longer incubation of seeds from different stages showed more intense staining of mature (20 daa) embryos and weaker staining of more immature (12 daa to 16 daa) embryos, but the same spatial pattern of expression was always observed.

To define further the correlation between the increase in GUS activity and ABA, immature seeds were removed from the capsule and incubated overnight with or without ABA (10^{-4} M). After incubation, all samples were assayed directly for GUS activity. Table 2 shows that the pBM1170-transformed seeds show no difference in the level of GUS activity after incubation with or without ABA. Seeds from the pBM1130 transformants, however, exhibited a threefold increase in GUS activity when incubated with ABA.

Table 2. β-Glucuronidase Activity in Seeds from Transgenic Tobacco Treated with ABA

Construction	Treatment	Activity
pBM1170	−ABA	29
pBM1170	+ABA	33
pBM1130	−ABA	50
pBM1130	+ABA	160
pBM3110	−ABA	0
pBM3110	+ABA	0

Activity and constructs are the same as in Table 1, with the addition of the Em-GUS construction in the reverse orientation (pBM3110). Light tan-colored seeds (12 daa to 14 daa) were dissected from the capsule and incubated overnight on a hormone-deficient nutrient medium (Murashige and Skoog, 1962) with (+) or without (−) 10^{-4} M ABA at 25°C in the dark.

5′ Deletion Analysis of the Em Promoter

We next attempted to determine, using our transient assay, the sequences between −554 and +92 that were involved in the ABA response. An exonuclease III deletion series was prepared to define these sequences more specifically (see Methods). Analysis of this deletion series in the transient assay system demonstrated a proportional decrease in the total GUS activity as the promoter fragment decreased from −554, either in the presence or absence of ABA. Figure 2 shows the results of transient assays for selected deletion endpoints. GUS activity of a 5′ deletion to −168 demonstrated two main points: (1) a reduction in overall activity, which was the characteristic response when sequences 3′ to −554 were deleted, and (2) a normal ABA induction (i.e., 15-fold to 20-fold). In contrast, a 5′ deletion to −106 was dramatically less active and responsive to ABA (i.e., threefold). Hence, the 62-bp region between −168 and −106 was likely to contain at least part of an ABA response element.

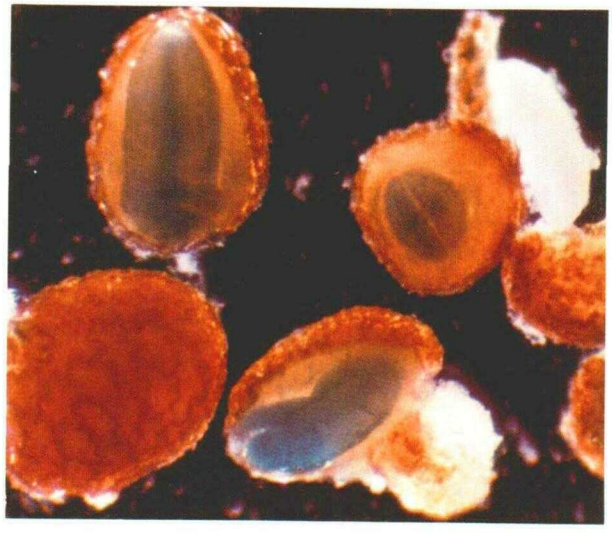

Figure 1. Histochemical Staining for GUS Activity in Transgenic Tobacco Seeds.

Seeds (20 daa) from transgenic tobacco containing the Em-GUS construction (pBM1130) were removed from the capsule, cut with a scalpel, and stained for 1 hr to 2 hr as described (see Methods). GUS activity (blue stain) is detected only in embryos.

Within this 62-bp fragment of the Em gene, we noticed an 8-bp perfect match (CGAGCAGG) between Em (−125 to −118) and a 5′ sequence (−225 to −217 from the translation start) from another ABA-regulated wheat gene, a 7S seed globulin, triticin (J. Litts, unpublished observations). Also, 7 of these 8 bp were found in the 5′ sequence of a seed-specific soybean gene, the α′ subunit of β-conglycinin (Chen, Schuler, and Beachy, 1986). Extending from this perfect 7-bp match in the 5′ regions of the soybean and wheat genes revealed a 24-bp segment that shared a 79% homology between the Em (from −156 to −133) and β-conglycinin (from −219 to −194) genes. A 50-bp oligonucleotide derived from the Em sequence (−152 to −103) containing this 24-bp region was synthesized and fused in either of two orientations, 5′ to a truncated (−90) 35S promoter. These constructs were introduced into rice protoplasts to determine whether they were responsive to ABA. Figure 3 shows that the 50-bp oligonucleotide fused to the truncated 35S promoter resulted in a twofold to threefold induction of GUS activity in either orientation when incubated with ABA.

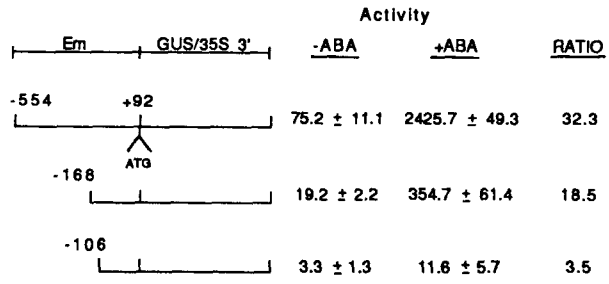

Figure 2. GUS Activity in Protoplasts Using Selected 5′ Deletions of the Em Gene.

Deletion endpoints −168 and −106 represent plasmids pBM113Δ220.9 and pBM113Δ240.13, respectively. Plasmids were transformed into rice protoplasts, and transient expression was analyzed after 18 hr. Plasmid pBM113Kp (Marcotte, Bayley, and Quatrano, 1988) represents full Em promoter activity. Activity is in picomoles of 4-methylumbelliferone per microgram of protein per hour. Errors represent standard deviation of triplicate samples within a single experiment. Experiments were repeated a minimum of three times with similar results. Ratio is the activity in the presence of ABA (+ABA) divided by the activity in the absence of ABA (−ABA). There was no GUS activity in mock-transformed protoplasts.

Post-transcriptional Regulatory Signals

Williamson and Quatrano (1988) demonstrated that expression of Em in wheat embryos is modulated by both transcriptional and post-transcriptional controls. Reduction in the level of GUS expression in response to ABA when using only a 50-bp sequence of the Em 5′ region, as compared with the response using the minimal Em 5′ region for a normal ABA response (260 bp, from −168 to +92), could be due, at least in part, to removal of Em sequences involved in post-transcriptional control (e.g., the 5′-untranslated sequence). To further investigate possible post-transcriptional component(s) of ABA regulation of Em expression, chimeric genes containing the 5′-untranslated Em sequence (+6 to +86) and/or an Em 3′ fragment were constructed, introduced into rice protoplasts, and assayed as described. Table 3 shows that a truncated 35S promoter with its own 3′ end is about fivefold less active in the rice protoplasts than the full promoter with enhancers (Odell et al., 1988). Like the full promoter, it is not responsive to ABA. Substitution of the Em 3′ end for the 35S 3′ end gave essentially identical activities.

However, Table 3 also shows that insertion of the +6 to +86 fragment of the Em gene between the truncated 35S promoter and the ATG codon of GUS resulted in two separable alterations in expression. In the absence of ABA, inclusion of the Em leader sequences restored GUS activity to greater than the level of the longer, full-length 35S promoter, a 10-fold enhancement over the truncated 35S promoter. In the presence of ABA, a small but reproducible increase of GUS activity was observed with the insertion of the 81 bp of the 5′-untranslated region, but not as strong a response as seen with the 50-bp sequence shown in Figure 3. Finally, Table 3 shows that, although the Em 3′ region had no effect with the 35S promoter alone, when both the +6 to +86 fragment of the Em gene and the Em 3′ region were present in the same construct, an overall decrease in activity was observed. Apparently, the Em 3′ fragment can interact with the Em 5′-untranslated region but not the 35S promoter sequences.

DISCUSSION

Several previous studies have indicated that results from expression analyses of 5′ regulatory sequences in transient assays are similar to those from transgenic experiments (e.g., Ellis et al., 1987; Walker et al., 1987) and that some promoters from monocots can function normally in dicots such as tobacco (e.g., Robert, Thompson, and Flavell, 1989). Regulation of GUS expression in transgenic tobacco seeds by the −554 to +92 fragment of the ABA-regulated wheat Em promoter shows the same temporal and spatial characteristics as expression of Em in developing wheat grains in three respects: (1) expression is confined to the embryo of the tobacco seed, not the endosperm or seed coat; (2) accumulation of activity is only in mature embryos, at the approximate time during late seed development when endogenous ABA levels peak in most angiosperms (c.f., Quatrano, 1987); and (3) precocious accumulation of GUS activity can be demonstrated in isolated immature seeds when cultured in the presence of ABA. These results from transgenic tobacco confirm our report using the rice transient assay that identified the

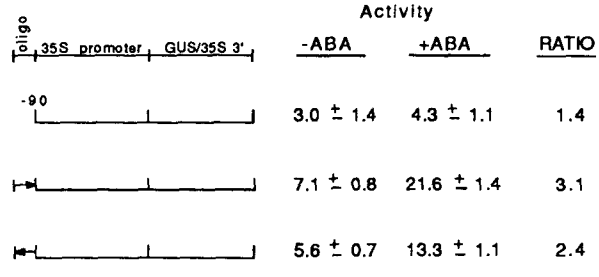

Figure 3. GUS Activity in Protoplasts Using 50 bp from a 5′ Region of the Em Gene.

The −152 to −103 region of the Em promoter was placed 5′ in both orientations of a −90 35S promoter-GUS chimeric gene. (Orientation is designated by the arrows.) Transformed protoplasts were analyzed after 18 hr. There was no GUS activity in mock-transformed protoplasts. Activity units, ratio, and reproducibility are as in Figure 2.

Table 3. GUS Activity in Protoplasts Using Fragments from the 5′-Untranslated and 3′ Regions of the Em Gene

Promoter	3′ Region	Activity −ABA	Activity +ABA
35S	35S	21.07 ± 2.51	28.55 ± 1.01
35S*	35S	4.12 ± 0.04	5.19 ± 0.64
35S*	Em	4.21 ± 0.60	3.36 ± 0.25
35S* + Em Leader	35S	48.08 ± 0.55	102.87 ± 10.91
35S* + Em Leader	Em	18.04 ± 2.28	40.04 ± 8.16

Sequences representing 81 of the 86 bases of the Em 5′-untranslated leader (+6 to +86) and a 442-bp TaqI fragment of the Em 3′ region were used alone or in combination in constructs containing a complete or −90 35S promoter (35S*) and GUS. Transformed protoplasts were analyzed after 18 hr. There was no GUS activity in mock-transformed protoplasts. Activity units and reproducibility are as in Figure 2.

same 646-bp fragment from the wheat Em 5′ region as containing an ABA-responsive element (Marcotte, Bayley, and Quatrano, 1988). Also, the hormonally regulated Em gene from wheat extends the list of monocot promoters that have been shown to be correctly regulated in the dicot tobacco. The relatively low level of induction seen with overnight incubation of the in vitro cultured tobacco seeds with ABA, as compared with the levels in overnight incubation of protoplasts in the transient assay, may be due to limited exposure of internal embryo cells of the tobacco seed to exogenous ABA, as well as to the sensitivity of this fragment of the Em gene to ABA in a heterologous, transgenic system.

Results from our 5′ deletion analysis of the 646-bp Em fragment in the rice transient assay indicate a decrease in overall transcriptional activity when sequences 5′ to −106 are removed, and that a 62-bp region between −168 and −106 contains sequences that are involved in the specific induction by ABA. The former result may be indicative of nonspecific transcriptional enhancer sequences. The segment of the Em 5′ region between −554 and −106 contains three sections rich in A/T and several copies of a 6-bp repeat (PuCACGPy) (J. Litts, unpublished observations). Bustos et al. (1989) have recently shown that an A/T-rich region of the β-phaseolin gene linked to GUS promoted strong expression of GUS in a number of different tissues of transgenic tobacco. The A/T-rich and repeat sequence motifs in the Em gene are presently being tested for enhancer activity. Figure 4 compares sequences in the 62-bp region between −168 and −106 with other ABA- and seed-regulated genes showing a few of the genes and their conserved regions. Obviously, the importance of these sequences will await further functional and structural analysis of these and similar promoters.

A class represented by box Em2 at −125 includes a 6-bp perfect match in two ABA/seed-regulated 5′ regions from wheat (Em and triticin). Seven of the 8 bp were found in the 5′-flanking region of α′ β-conglycinin gene from soybean, which is expressed in embryos but is not inducible by ABA in vivo (Bray and Beachy, 1985). Six of these 7 were also found in Rab21, an ABA/seed-regulated promoter from rice (Mundy and Chua, 1988). Furthermore, expanding the region around the perfect 7-bp match between β-conglycinin and Em2 to 24 bp shows a 79% homology. Whether the conserved sequence represented by the Em2 class (CGAGCAg) might be involved in seed specificity and/or the ABA response is presently being tested.

The class represented by box Em1 shows a 9-bp consensus sequence (ACGTGccgC), which is repeated at −149 (Em1a) and at −94 (Em1b) in the Em 5′ region. It is also found at −183 in the Rab21 5′ region (Mundy and Chua, 1988). In a sequence analysis of auxin-regulated genes expressed in vegetative soybean tissue, Ainley et al. (1988) and McClure et al. (1989) have identified conserved sequences in these 5′ regions. The conserved sequences from the 5′ regions of these auxin-regulated genes, as well as the ethylene-regulated (Broglie et al., 1989) and GA-regulated (Huttly, Martienssen, and Baulcombe, 1988) genes, are completely different from the conserved sequences in the 5′ regions from the ABA-regulated genes.

Both Em1a and Em2 boxes were included in the 50-bp region that was synthesized and linked (in both orientations) 5′ to a truncated CaMV 35S promoter. Both orien-

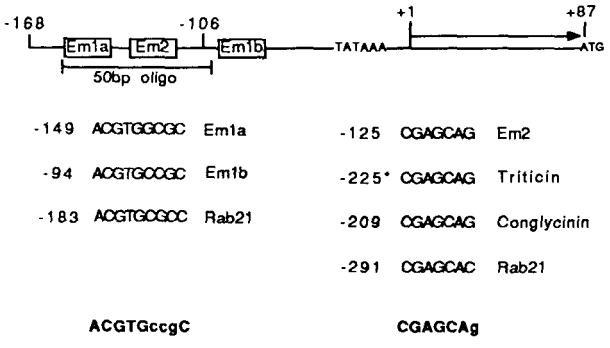

Figure 4. Schematic Representation of the Em Gene and Sequence Comparisons.

Endpoints for the deletions in Figure 2 are marked (−168 and −106). The region corresponding to the 50 bp (−152 to −103) analyzed in Figure 3 is also marked. The number designations refer to the 5′ base of the sequence shown, and all sequences are numbered with respect to the start of transcription except Triticin (*), which is with respect to the start of translation. Rab21 sequences are from Mundy and Chua (1988), and conglycinin sequences are from Chen, Schuler, and Beachy (1986). Proposed consensus sequences for the Em1a/b and Em2 boxes are given at the bottom of the figure in boldface.

tations were shown to confer ABA inducibility on this viral promoter, indicating that an ABA response element was present in this sequence. Hence, activity of this 50-bp sequence in either orientation closely resembles some properties of the hormone response elements found in animal systems (c.f. Evans, 1988; Burnstein and Cidlowski, 1989). However, the level of ABA induction with this oligonucleotide was fivefold to 10-fold less than that observed with the −168 fragment. Because the −168 fragment included the 5′-transcribed/untranslated region and previous results from this lab indicated a post-transcriptional level of regulation in the control of Em expression by ABA (Williamson and Quatrano, 1988), we decided to test the role of other sections of the Em gene in ABA regulation.

Post-transcriptional effects have been shown to be mediated by various parts of genes, including 5′-untranslated (Sleat et al., 1987) and 3′-untranslated regions (Wreschner and Rechavi, 1988; Ingelbrecht et al., 1989). It is clear that the Em 3′ region does not influence GUS expression in the rice transient assay when used in conjunction with the truncated 35S promoter. The Em 3′ fragment does have an effect on GUS expression in constructs containing the Em 5′-untranslated region. The nature of this interaction is being investigated further.

The 5′-untranslated sequence from the Em gene inserted between the 35S promoter and GUS clearly had a major effect, increasing GUS expression in the rice protoplasts 10-fold without added ABA. The −106 fragment, which includes the TATAAAAA at −49 and the +6 to +86 region, does not lead to enhanced GUS expression (Figure 2). This result suggests that this fragment does not contain an enhancer function, but may play a role in stabilizing or increasing the efficiency of translation of the mRNA. Only with an additional 62 bp of the Em 5′-untranslated region (to −168), which contains the putative ABA response element, or the truncated CaMV promoter, which contains more general enhancer sequences, does the +6 to +86 fragment have an enhancing effect on the expression of GUS. Regardless of the mechanism involved, this result, combined with the data using the 50-bp 5′ sequence alone, suggests that the minimal 5′ fragment that has been shown to give the full response to ABA (i.e., −168 to +92) may have two functional components: a specific response element (−152 to −103) and an element (+6 to +86) that quantitatively increases the ABA response.

We have recently shown that the Em gene is expressed in rice suspension cultures (S. Schmitz, unpublished observations), as well as in wheat seedlings in response to exogenous ABA (Berge, Bartholomew, and Quatrano, 1989) and desiccation (S. Berge, unpublished observations). These results suggest that the Em gene is not expressed only in embryo tissue in response to ABA, as was previously reported (Williamson and Quatrano, 1988). These more recent results show a pattern of Em expression similar to that of other ABA-regulated genes isolated from developing grains of rice (Mundy and Chua, 1988), barley (Chandler et al., 1988), and maize (Gomez et al., 1988), in that numerous tissues can respond to ABA as measured by the enhanced expression of Em. We are interested in determining whether the response of this gene to ABA and various stresses in different tissues requires the same or different regions of the 5′ and 3′ regulatory regions. For example, is the 5′ region of the Em gene involved in the response to ABA the same region that is regulating the response to water stress and desiccation? Do these regions respond to these different signals similarly in embryo and vegetative tissue? To answer these questions, various parts or boxes of the 5′ region of the Em gene identified in this study will be combined with untranslated 5′ and 3′ fragments of the Em gene and introduced into transgenic seeds/seedlings to determine their functional role in tissue-specific expression in response to water stress and the application of exogenous ABA.

METHODS

Construction of Plasmids

All nucleic acid manipulations were carried out using standard procedures (Maniatis, Fritsch, and Sambrook, 1982; Ausubel et al., 1987).

Plasmids pBM1170, pBM1130, and pBM3110 are derivatives of pBIN19 (Bevan, 1984) into which the promoter-GUS-35S 3′ cassettes from pBM117, pBM113Kp, and pBM311 (Marcotte, Bayley, and Quatrano, 1988), respectively, have been cloned as HindIII/EcoRI fragments.

Plasmid pBM113 has been described previously (Marcotte, Bayley, and Quatrano, 1988). Plasmids pBM113Δ220.9 and pBM113Δ240.13 are exonuclease III-generated deletions of plasmid pBM113 with deletion endpoints at −168 and −106, respectively. The exonuclease III deletion series was created using Erase-a-Base from Promega-Biotec. Plasmid pBM113 was linearized using HindIII, after which the HindIII site was protected from exonuclease III digestion by filling in the overhang with α-phosphorothioate deoxynucleotide triphosphates. Secondary cleavage with BglII exposed a site 1328 bases from the ATG codon that was sensitive to exonuclease III digestion. Digestion was performed at 37°C, and aliquots were removed at 10-sec intervals. Plasmid pBM113Δ220.9 is isolate 9 from the 220-sec timepoint. Similarly, pBM113Δ240.13 is isolate 13 from the 240-sec timepoint. Deletion endpoints were defined by sequence analysis (Zagursky et al., 1985).

Two oligonucleotides were synthesized and annealed to create a 50-bp region of the Em promoter from −152 to −103. The oligonucleotides were synthesized on an ABS synthesizer, and half of an EcoRV site was included at each end of the oligo (ATC at the 5′ end and GAT at the 3′ end, resulting in a 56-bp oligonucleotide). Plasmids pBM172 and pBM183 contain the oligonucleotide 5′ of the truncated (−90) 35S promoter. These plasmids were created by first ligating the oligonucleotide into the

EcoRV site at −90 in the 35S promoter. Orientation of the single oligonucleotide inserts was determined by sequencing, and two isolates were retained, one in each orientation. Digestion with SmaI, followed by partial digestion with EcoRV, allowed the isolation of a 157-bp fragment containing the truncated 35S promoter, immediately preceded by the oligonucleotide. These fragments were ligated into pBM120, which had been cut with SalI, and the ends rendered blunt. Plasmid pBM172 contains the oligonucleotide in the forward orientation and plasmid pBM183 contains the oligonucleotide in the reverse orientation.

Plasmid pBM152 contains the cauliflower mosaic virus (CaMV) 35S promoter followed by GUS and the CaMV 35S 3′ region. The GUS gene was isolated from pRAJ275 (Jefferson, 1987) as an SalI/EcoRI fragment, the SalI site was made blunt, and the fragment was cloned into SmaI/EcoRI-cut pUC18 (pBM119). The 35S 3′ region was isolated as a 200-bp EcoRI fragment from pDH51 (Pietrzak et al., 1986) after cleavage with NcoI/SphI, filling in the ends, and re-ligation. This fragment was cloned into the EcoRI site of pBM119 at the 3′ end of GUS to yield pBM120. The 35S promoter was isolated from pDH51 as a blunt-ended EcoRI/SmaI fragment and was cloned into the SalI site (blunt-ended) of pBM120 to yield pBM152. An analogous construct (pBM173) contains a truncated 35S promoter region prepared by ligation of a 101-bp EcoRV/SmaI fragment from pDH51 into similarly prepared pBM120.

Plasmid pBM185 is a derivative of pBM173 in which the 35S 3′ region has been replaced by the Em 3′ region. The Em 3′ region was isolated as a 442-bp TaqI fragment from pEAS41.8 (J. Litts, unpublished observations), and the ends were made blunt. The 35S 3′ region was removed from the 3′ end of GUS by cleavage with EcoRI, after which the EcoRI sites were filled in. Ligation of the blunt-ended TaqI fragment into the blunt-ended EcoRI site creates EcoRI sites at each end of the Em 3′ fragment. Plasmid pBM186 is a derivative of pBM173 in which the Em 5′-transcribed, nontranslated sequences have been placed between the 35S transcriptional start site and the GUS gene. Oligonucleotide-directed mutagenesis was used to make a single base pair change in the context around the Em ATG (CAATGG to CCATGG) to create an NcoI site. The 5′-untranslated sequences were isolated as an 80-bp XhoI/NcoI fragment. The XhoI site was filled in and was cloned into pBM173 that had been cleaved with BamHI, the ends filled in, and secondary cleavage with NcoI. This results in a construct that will produce a messenger RNA that has a 98-base leader containing 81 bases of the Em 5′-untranslated sequences. Plasmid pBM187 is a derivative of pBM186, in which the 200-bp EcoRI fragment containing the 35S 3′ region has been replaced with the 449-bp EcoRI fragment containing the Em 3′ region (from pBM185).

Production of Transgenic Plants

Binary constructs pBM1170, pBM1130, and pBM3110 were mobilized into the *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983) using HB101 (pRK2013) as the helper *Escherichia coli* strain (Figurski and Helinski, 1979). Leaf disc transformation was as described by Horsch et al. (1985). Regenerated shoots were selected on medium containing 100 μg/mL kanamycin. After rooting, transgenic plantlets were transferred to soil and grown in a growth room (85% relative humidity) under a 14-hr light (21°C) and 10-hr dark (18°C) photoperiod.

Analysis of GUS Activity in Protoplasts and Transgenic Plants

Transformation of rice protoplasts and transient expression analyses have been previously described (Marcotte, Bayley, and Quatrano, 1988). Analysis of GUS expression in transgenic plant tissues was as described by Jefferson (1987).

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