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Functional Dissection of an Abscisic Acid (ABA)-Inducible Gene Reveals Two Independent ABA-Responsive Complexes Each Containing a G-Box and a Novel *cis*-Acting Element

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To elucidate the mechanism by which abscisic acid (ABA) regulates gene expression, the promoter of the barley ABA-responsive *HVA22* gene has been analyzed by both loss- and gain-of-function studies. Previous reports indicate that G-box sequences, which are present in genes responding to a variety of environmental and physiological cues, are involved in ABA response. However, our data suggest that G-box sequences are necessary but not sufficient for ABA response. Instead, an ABA response complex consisting of a G-box, namely, ABRE3 (GCCACGTACA), and a novel coupling element, CE1 (TGCCACCGG), is sufficient for high-level ABA induction, and replacement of either of these sequences abolishes ABA responsiveness. We suggest that the interaction between G-box sequences, such as ABRE3 in the *HVA22* gene, and CE-type sequences determines the specificity in ABA-regulated gene expression. Our results also demonstrate that the ABA response complex is the minimal promoter unit governing high-level ABA induction; four copies of this 49-bp-long complex linked to a minimal promoter can confer more than 100-fold ABA-induced gene expression. In addition to ABA response complex 1, composed of ABRE3 and CE1, the *HVA22* promoter contains another ABA response complex. The ABA responsiveness of this ABA response complex 2 relies on the interaction of a G-box (ABRE2; CGCACGTGTC) with another yet unidentified coupling element. These two complexes contribute incrementally to the expression level of *HVA22* in response to ABA.

INTRODUCTION

Abscisic acid (ABA) has been shown to mediate many physiological and developmental processes throughout the life cycle of plants. Several approaches have been adopted to study the molecular mechanism of ABA action involved in these processes. One of them is to isolate and characterize the ABA receptors, the presumable primary site of action for ABA response. Some progress has been made in this field, suggesting the presence of both extracellular and intracellular reception sites for ABA (Allan et al., 1994; Anderson et al., 1994; Gilroy and Jones, 1994; Schwartz et al., 1994). Another approach takes advantage of ABA response mutants from which some of the genes involved in the signal transduction pathway of ABA response have been cloned. One of these genes encodes a transcription factor, Viviparous1 (VP1), that has been shown to mediate ABA-regulated gene expression (McCarty et al., 1991). Recently, the Arabidopsis ABA insensitive 1 (*ABI1*) gene has been cloned, and it is suggested that this gene encodes a protein phosphatase involved in ABA signal transduction (Leung et al., 1994; Meyer et al., 1994).

Because it has been well established that ABA regulates the expression of a variety of genes, the third approach for studying the mechanism of ABA action is to identify the *cis*-acting elements necessary and sufficient for ABA response and to isolate the *trans*-acting factors interacting with these DNA sequences. Characterization of the genes encoding those *trans*-acting factors would move us one step closer to the initial ABA response site, and by doing so, it is hoped that the ABA signal transduction pathway(s) could at least be partially deciphered.

Initially, the 5′ upstream sequences of many ABA-responsive genes were compared, and the conserved sequences were believed to be the putative ABA-responsive elements (Marcotte et al., 1989; Mundy et al., 1990). Transient expression studies with various wheat *Em* promoter sequences driving the β-glucuronidase (*GUS*) gene in rice protoplasts suggested that a 260-bp fragment (−168 to +92) of the *Em* gene resulted in a 15-fold induction of GUS activity in the presence of ABA (Marcotte et al., 1989). A 75-bp fragment of this gene, when fused in either direction to a truncated cauliflower mosaic virus 35S promoter, gave greater than 10-fold ABA induction (Guiltinan et al., 1990). Using a similar system, Mundy et al. (1990) reported that a promoter fragment between −294 and −52 of the rice ABA-responsive *Rab16A* gene was sufficient to confer ABA-dependent expression in rice protoplasts. Skriver

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et al. (1991) demonstrated that six copies of the sequence GTACGTGGCGC conferred sixfold ABA induction to a 35S minimal promoter. This sequence and its homologs have been designated as ABRE (ABA Response Element) (Guiltinan et al., 1990; Skriver et al., 1991; Shen et al., 1993). However, it is perplexing that the so-called ABRE is very similar to the G-box that, as has been pointed out by Guiltinan et al. (1990), is present in a variety of genes that are responsive to other environmental and physiological cues, such as light (Giuliano et al., 1988; Schulze-Lefert et al., 1989), auxin (Liu et al., 1994), jasmonic acid (Mason et al., 1993), and salicylic acid (Qin et al., 1994). One possibility is that the sequences flanking the ACGT core determine signal response specificity. This is supported by evidence that binding patterns to the proteins in nuclear extracts are affected by sequences flanking CACGTG in a gel mobility shift assay (Williams et al., 1992). Furthermore, different cloned ACGT-core–binding basic leucine zipper (bZIP) proteins interact with G-box–like sequences with different affinities (Armstrong et al., 1992; Schindler et al., 1992b; Williams et al., 1992; Izawa et al., 1993; Foster et al., 1994). Another possibility is that the specificity is dependent on the coupling of an ACGT-core sequence with another *cis*-acting element. Evidence for this specificity is provided by Schulze-Lefert et al. (1989) who have shown that a light-responsive unit is composed of an ACGT-core–containing sequence and a non-ACGT *cis*-acting element.

To date, a thorough analysis of an ABA-responsive promoter has not been performed. As a result, the minimal single-copy promoter sequence necessary and sufficient for ABA response has not been determined. Our goal was to determine the promoter context in which a single-copy ABA response sequence confers a high level of gene expression. Furthermore, we would like to define the sequence(s) that governs the ABA response specificity, given the puzzle that G-box sequences are present in non-ABA–responsive promoters. In earlier studies, we have isolated a single-copy ABA-responsive barley gene, *HVA22*, whose promoter has been analyzed by delivering DNA constructs into intact barley aleurone layers via the particle bombardment technique (Klein et al., 1987; Shen et al., 1993). 5′ Deletion analyses indicate that the promoter sequence up to −282 is sufficient for ABA response and three G-box–like sequences are present in this region (Shen et al., 1993). In addition, intron 1 of the *HVA22* gene is also required for high-level ABA induction of *HVA22* expression (Shen et al., 1993). A G-box located at the −90 region (formerly designated OP-G box; renamed ABRE3) might also be involved in the ABA response. We have suggested that a complex consisting of at least two elements in the promoter region and the first intron is essential for high-level ABA induction of *HVA22* expression (Shen et al., 1993).

5′ Deletion studies cannot define all *cis*-acting elements necessary for a signal response. In a response complex, disruption of one of the components might completely abolish the activity of a promoter. For instance, a gibberellin response complex consists of several *cis*-acting elements, including the gibberellin response element (GARE), Opaque2 binding sequence (O2S)/endosperm box, and two other elements. Deletion of either GARE or O2S essentially abolishes promoter activities (Lanahan et al., 1992; Rogers and Rogers, 1992). Therefore, we conducted linker–scan analyses and gain-of-function studies to define the minimal sequence that drives high-level ABA induction in the native promoter. We report here that not all G-box sequences are involved in the ABA response of the *HVA22* promoter. Those that are involved are not sufficient for ABA response if they are present alone. Instead, a basic ABA response complex is found to consist of a G-box and a novel coupling element. The coupling of a G-box with another *cis*-acting element could be the basis of signal response specificity and transcription strength determination. We also show here that more than one ABA response complex is present in the *HVA22* gene and that these complexes contribute incrementally to the expression level of the gene in response to ABA.

RESULTS

Two of the Three G-Box Sequences in the *HVA22* Gene Promoter Are Involved in ABA Responsiveness

HVA22 appears to be a single-copy gene localized on barley chromosome 1 (Shen et al., 1993). RNA gel blot analyses indicated that its expression is regulated by ABA and environmental stress. Furthermore, this gene can also be induced by the protein synthesis inhibitor cycloheximide (Shen et al., 1993), a phenomenon that has been observed with many genes encoding transcription factors (Milbrandt, 1986; Shaw et al., 1989). Sequence comparison indicated that the *HVA22* gene product is highly homologous to that of a human gene, *DP1*, that is

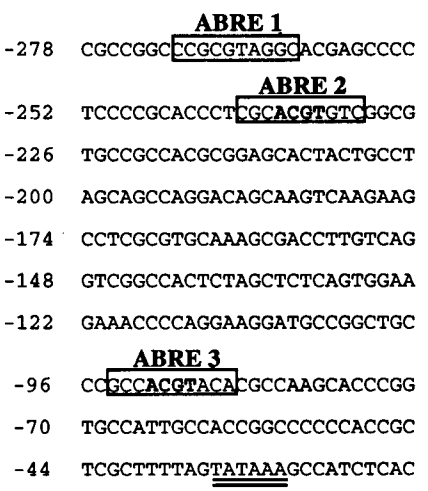

Figure 1. The TATA-Proximal Sequence of the *HVA22* Gene Promoter.

The ABRE1, ABRE2, and ABRE3 sequences are enclosed in boxes, and the TATA box is double underlined.

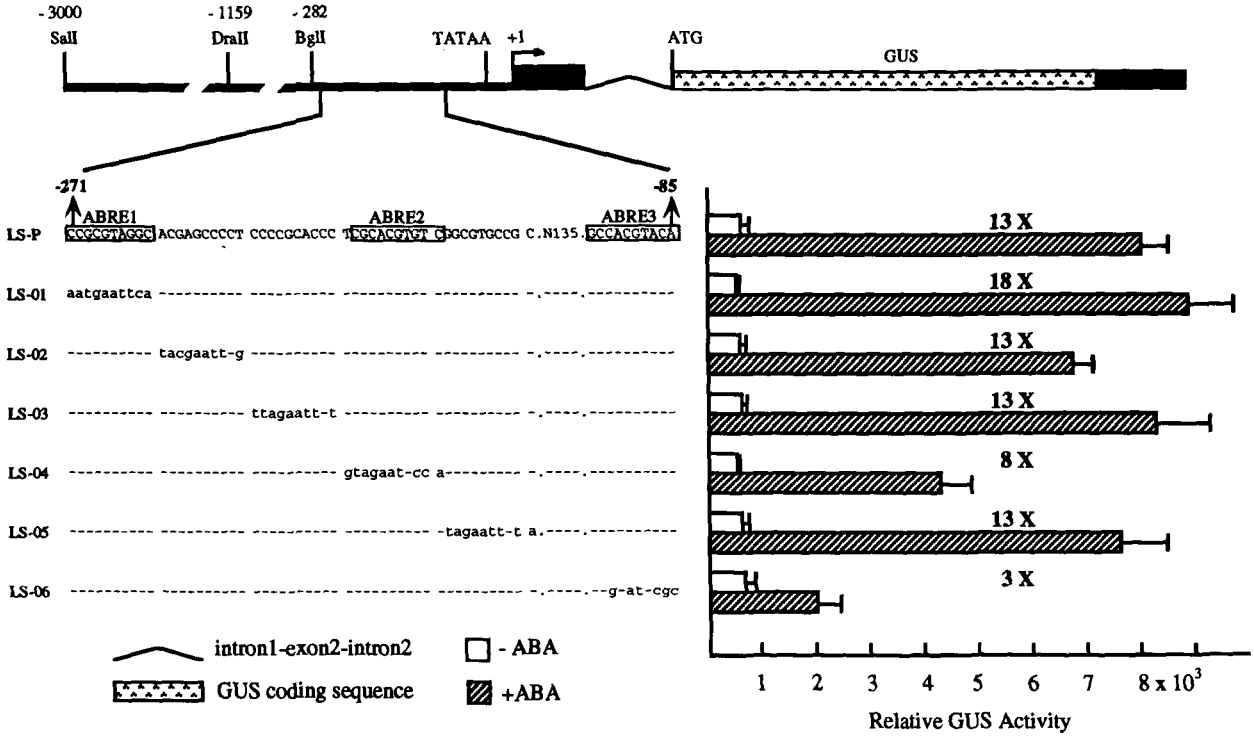

Figure 2. Only Two of the Three G-Box Sequences in the Barley *HVA22* Gene Promoter Are Involved in ABA Responsiveness.

A schematic diagram of the test construct is shown at the top. The thick black line at left represents the *HVA22* promoter region. The thin black angled line indicates the position of the intron1–exon2–intron2 fragment of *HVA22* inserted between the 5′ untranslated sequence (solid box) and the *GUS* coding sequence (box with clusters of dots). The 3′ region (black bar to the right of the *GUS* coding sequence) was from the *HVA22* SphI-SphI genomic fragment, including the polyadenylation sequence (AATAAA). LS-P contains the 3-kb *HVA22* promoter with the BglI site at −280 destroyed by a 17-bp insertion (see Methods). The number of the sequence is relative to the second transcription start site (Shen et al., 1993). The promoter sequence of interest is shown to the right of the wild-type construct LS-P. Other linker-scan constructs (LS-01 to LS-06) are similar to LS-P, except for the base substitutions shown in lowercase letters. Dashes indicate the wild-type sequence as shown in LS-P. To the right are the levels of GUS activities expressed by each construct. White boxes represent relative GUS activity of the samples from embryoless half-seeds incubated in the absence of ABA (−ABA), and hatched boxes represent those from embryoless half-seeds incubated in the presence of 2 × 10^{-5} M ABA (+ABA). Relative GUS activity of each construct, calculated as described in Methods, is the mean of four replicas. Error bars indicate the standard error of each set of replicas. X indicates fold increase.

believed to be involved in human colorectal tumorigenesis (Joslyn et al., 1991). These interesting features encouraged us to isolate and analyze the promoter of this barley gene. The promoter sequence proximal to the TATA box is shown in Figure 1. The *HVA22* promoter contains three G-box sequences, designated ABRE1, ABRE2, and ABRE3 (Figure 1). We have shown previously that the region containing ABRE1 and ABRE2 is necessary for the ABA response of *HVA22* (Shen et al., 1993). In addition, ABRE3 is highly similar to the sequence important for ABA induction of other genes (Guiltinan et al., 1990; Mundy et al., 1990). Therefore, detailed linker-scan analyses were conducted across the region (−281 to −211) containing the ABRE1 and ABRE2 sequences in addition to the ABRE3 element (−94 to −85). The original *HVA22* promoter sequence was replaced at 10- or 11-bp intervals. Analyses of these mutants using a particle bombardment-mediated transient expression system indicated that the effect of the ABRE3 mutation is most significant. As illustrated in Figure 2, the mutation of this sequence led to a 75% reduction in ABA-inducible GUS activity and a decrease of induction from an average of 15-fold to threefold (Figure 2, construct LS-06). In addition, the mutation of ABRE2 reduced the level of expression from the *HVA22* promoter to less than 50% of that obtained from a wild-type promoter (LS-04). No significant effect was observed when mutations were introduced in the other regions between −281 and −211, including ABRE1 (LS-01, LS-02, LS-03, and LS-05).

Double Mutation of ABRE2 and ABRE3 Essentially Abolishes ABA Responsiveness of the *HVA22* Gene

Double mutation experiments were conducted with the 3-kb *HVA22* promoter, and the results are presented in Figure 3.

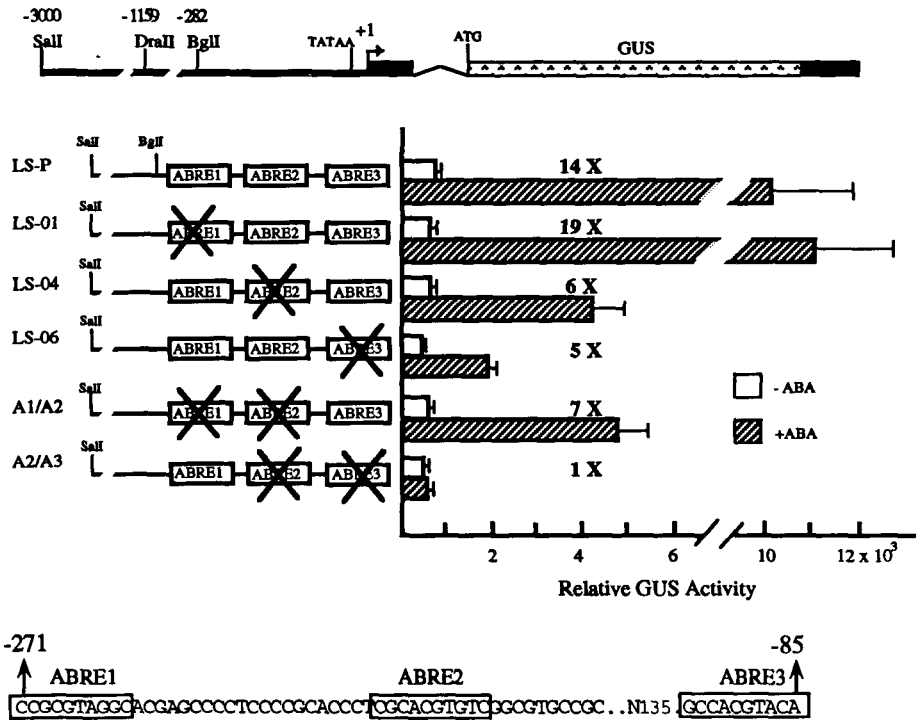

Figure 3. Double Mutation Analyses of ABRE1, ABRE2, and ABRE3 in the 3-kb *HVA22* Promoter.

The basic *HVA22* promoter is the same as shown in Figure 2, except the mutated ABRE is indicated with a strike (X). The promoter sequence containing ABRE1, ABRE2, and ABRE3 is shown at the bottom. N135 represents the distance between the two stretches of the sequence listed at bottom, that is, 135 bp. All other symbols are as given in the legend to Figure 2. Relative GUS activity of each construct, calculated as described in Methods, is the mean of four replicas. Error bars indicate the standard error of each set of replicas. X indicates fold increase.

As given above, the replacement of ABRE1 alone had no effect, whereas changing the ABRE2 sequence reduced the absolute level of expression and the fold induction by ABA (Figure 3, LS-01 and LS-04, respectively). The mutation of ABRE3 had an even greater negative effect on the response of *HVA22* to ABA (LS-06). The double mutation of ABRE1 and ABRE2 had a result similar to that of the ABRE2 single mutation (compare construct A1/A2 with LS-04), confirming that ABRE1 might not be necessary for ABA induction of *HVA22*. In contrast, when both ABRE2 and ABRE3 were mutated, little expression above the background level was obtained (A2/A3). This result suggests that both ABRE2 and ABRE3 are involved in the response of *HVA22* to ABA.

A 49-bp Fragment Containing ABRE3 Confers a High Level of ABA Induction to a Minimal Promoter

To test whether the sequence containing ABRE3 is able to confer ABA inducibility, the BsrFI-BsrFI (49-bp) fragment of *HVA22* was fused to the 5′ end of a truncated (−60) barley α-amylase (*Amy64*) promoter in both orientations. As shown in Figure 4, whereas the control (*Amy64* minimal promoter only) construct was not affected by ABA treatment (P = 0.16), the addition of the 49-bp ABRE3-containing element in either orientation resulted in a high level (24- to 38-fold) of induction (1C+ and 1C−, Figure 4). It is interesting that both the fold induction and the absolute level of GUS activity obtained from construct 1C+ were much higher than those from the 3-kb wild-type *HVA22* promoter (compare Figures 2 and 3 with Figures 4 and 5). It is possible that there are negatively regulating elements outside of the 49-bp region of the *HVA22* promoter and/or in the 5′ untranslated region of the *HVA22* gene.

To exclude the possibility that the DNA sequence of the *Amy64* promoter is involved in the ABA response, we replaced the *Amy64* promoter with the minimal promoter (−80) from *EP-B*, a barley cysteine protease gene (A. Mikkonen, I. Porali, M. Cercos, and T.-H.D. Ho, manuscript in preparation). This minimal promoter works as well as the *Amy64* promoter, resulting in more than 30-fold induction (data not shown). Comparison of the *EP-B* minimal promoter sequence with that of the *Amy64* showed no similarity between the two sequences except for the TATA box. Therefore, it appears that all the sequences essential for the ABA response of construct 1C+ reside in that 49-bp fragment and possibly in the intron as described later.

In this experiment, we also observed that the high level of ABA induction from construct 1C+ relies on the presence of

the intron1–exon2–intron2 fragment from the *HVA22* gene. Deletion of this fragment resulted in the decrease of ABA induction from 38-fold to only threefold (intronless construct, Figure 4). This study confirms our previous report that intron 1 is important for the ABA response of the native *HVA22* promoter (Shen et al., 1993). Introns are believed to be general enhancers for genes in monocotyledonous plants because their presence is important for the high level of gene expression in transient expression studies (for example, see Callis et al., 1987). Our previous and current studies argue that introns may also be involved in the response to ABA because the deletion of them led to a drastic decrease in ABA induction as well as in the absolute level of GUS activity (Figure 4 and Shen et al., 1993). Involvement of introns in hormone response has also been reported in mammalian systems (Moore et al., 1985; Sap et al., 1990; Berkowitz and Evans, 1992).

The duplication of the 49-bp fragment resulted in the increase of both fold induction and absolute GUS activities (Figure 4). Fold induction increased almost linearly as more copies of the fragment were added, and the 4C+ construct routinely resulted in a more than 120-fold induction by ABA (4C+, Figure 4). To our knowledge, this is the most dramatic ABA response among all the native or modified ABA promoters. Interestingly, the basal level of GUS activities obtained from the duplication constructs remained similar to that obtained from the single-copy construct. It is possible that the duplication of the sequence may promote the stable interactions between *trans*-acting factors binding to the ABA response element(s).

A G-Box and a Novel *cis*-Acting Element within the 49-bp Promoter Region Are Required for ABA Responsiveness

To determine the sequences within the 49-bp promoter region that govern ABA responsiveness of the fragment, linker-scan

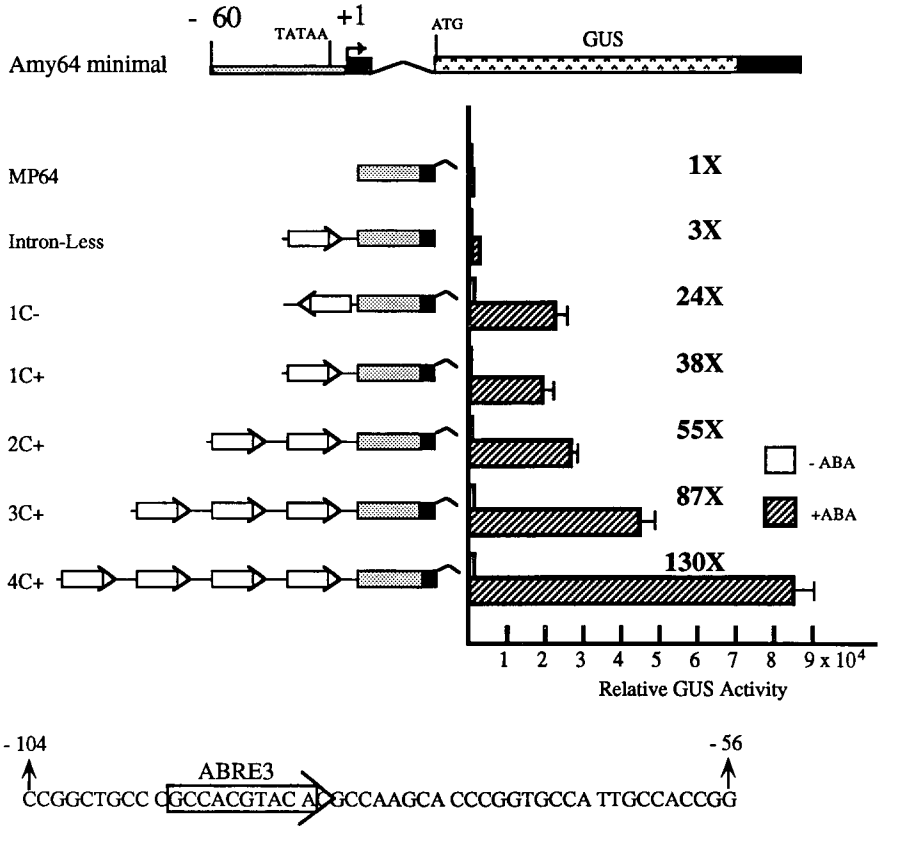

Figure 4. A 49-bp Fragment Containing ABRE3 Confers ABA Inducibility to a Minimal Promoter.

The minimal promoter (to −60) and the 5′ untranslated region (to +57) (stippled rectangle) from the barley *Amy64* α-amylase gene were fused to the 5′ end of the *HVA22* intron1–exon2–intron2 fragment in the construct IGU (see Methods). This minimal promoter (MP64) is not responsive to gibberellin or ABA. The 49-bp *HVA22* promoter fragment, shown at the bottom, was fused in either the positive (i.e., the same as in the native promoter) or negative orientation. The 2C+, 3C+, and 4C+ constructs contain two, three, or four tandem copies of the 49-bp sequence, respectively. The numbering of the 49-bp fragment is relative to the transcription start site of the *HVA22* gene. All other symbols are as given in the legend to Figure 2. Relative GUS activity of each construct, calculated as described in Methods, is the mean of four replicas. Error bars indicate the standard error of each set of replicas. X indicates fold increase.

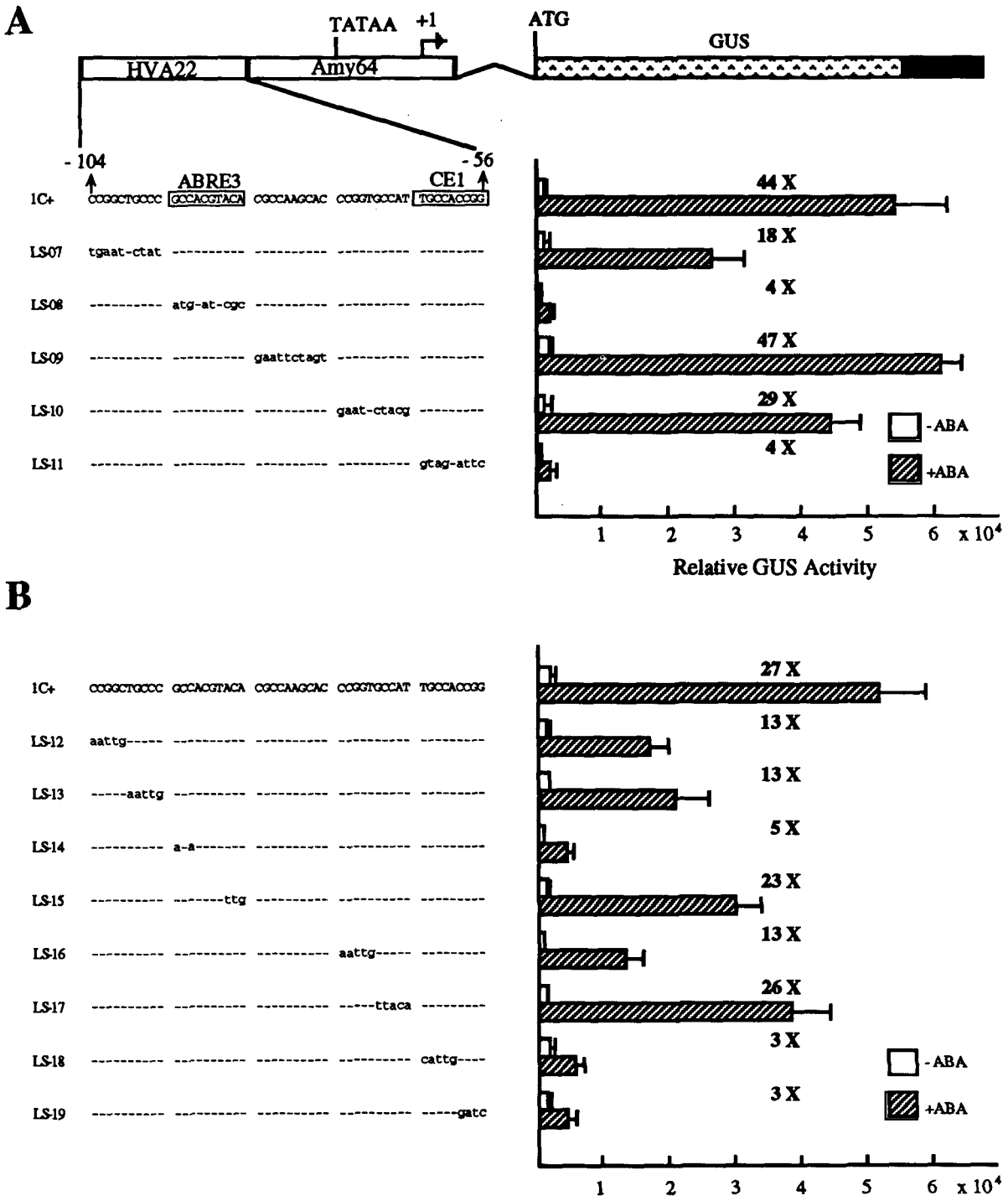

Figure 5. Linker–Scan Analyses of the 49-bp Region Define a Novel CE1 Element Involved in the ABA Response.

The numbering of this fragment is relative to the transcription start site of the *HVA22* gene.
(A) The promoter sequence was mutated at 10-bp intervals. The mutated bases are shown in lowercase letters.
(B) A more detailed analysis in which the promoter fragment was mutated at 5-bp intervals.
Symbols are as given in the legend to Figure 2. Relative GUS activity of each construct, calculated as described in Methods, is the mean of four replicas. Error bars indicate the standard error of each set of replicas. X indicates fold increase.

analysis was performed with construct 1C+. In Figure 5A, the promoter fragment was replaced at 10-bp intervals. The mutation of the third (LS-09, Figure 5A) 10-bp element had little effect on ABA responsiveness. A negative effect was observed with construct LS-10 in which the fourth 10-bp sequence was replaced. Similarly, the mutation of the first 10-bp sequence resulted in ~50% reduction in both fold induction and the absolute level of GUS activity (LS-07). The most drastic reduction was observed with two mutants, LS-08 and LS-11; the absolute level of GUS activities obtained from these constructs dropped to below 5% of that obtained with the wild type. Accordingly, fold induction decreased from 44-fold in the case of the wild type to only fourfold for these two mutants (Figure 5A). In construct LS-08, the ACGT-core–containing ABRE3 element was mutated, whereas in LS-11 the last 9 bp of the 49-bp promoter fragment was mutated. This 9-bp fragment represents a novel *cis*-acting sequence involving the ABA response and was designated CE1 (for Coupling Element).

A more detailed linker–scan analysis was conducted to confirm the aforementioned result and to further define the border of ABRE3 and CE1. In this study, parts of the 49-bp fragment were mutated at 5-bp intervals as illustrated in Figure 5B. In most of the constructs, all five base pairs were mutated, with the exception of the mutants that replaced ABRE3 (LS-14 and LS-15). In these two constructs, only sequences flanking the ACGT core were mutated because ACGT has been shown to be conserved among the G-box sequences present in ABA-responsive genes (Michel et al., 1993). Effects of mutations observed with this set of the 5-bp mutants were very similar to those of the 10-bp mutants. It is obvious that the replacement within either ABRE3 or CE1 almost completely abolished ABA responsiveness of the promoter (LS-14, LS-18, and LS-19, Figure 5B). Interestingly, the flanking sequence upstream of the ACGT core appears to be much more crucial than the one downstream of the core. The 2-bp mutation upstream of the ACGT core dramatically reduced ABA induction, whereas the 3-bp mutation downstream had much less effect. As a whole, this study supports the conclusion that the minimal ABA response complex in the barley *HvA22* gene consists of a G-box (ABRE3) and a novel *cis*-acting element (CE1). We designated this 49-bp fragment ABA response complex 1.

Sequences similar to CE1 are present in other ABA-responsive genes, such as maize *Rab17* (Vilardell et al., 1990) and rice *Rab16A* (Mundy and Chua, 1988), that are listed in Table 1. None of these elements have been tested to determine whether they are indeed involved in ABA responsiveness of those genes. In light of our data described previously, it would definitely be worthwhile to study whether these CE1-like sequences are the coupling elements of the ABA response complexes in those genes.

The *HVA22* Gene Promoter Contains Two ABA Response Complexes

Because our loss-of-function study indicates both ABRE2 and ABRE3 are involved in ABA induction of the *HVA22* gene (Figures 1 and 2), we reasoned that besides ABA response complex 1 containing ABRE3 and CE1 described in the previous section, there might be another ABA response complex in this promoter. To test this possibility, the promoter sequence between −276 and −101, which is located immediately upstream of ABA response complex 1 and contains ABRE1 and ABRE2, was fused to the *Amy64* minimal promoter. As shown in Figure 6, it appears that the addition of this 176-bp fragment conferred 14-fold ABA induction (construct CX2). Neither the upstream element (−276 to −231, CX2E1) nor the downstream element (−220 to −101, CX2E2) of this fragment alone could confer a significant level of ABA induction. The failure of the

Table 1. CE1-like Sequences Are Present in Other ABA-Responsive Genes

	CE1-like Element		G-Box		
Gene	Position	Sequence	Position	Sequence	Reference
HVA22	−64	TGC CACC GG	−95	GCC ACGT ACA	This study
Rab17	−191	GGC CACC GA	−148	CGT ACGT GTA	Vilardell et al. (1990)
Rab16A	−218	CAC CACC CG	−181	CGT ACGT GCG	Mundy and Chua (1988)
Rab16D	−111	GCC CACC TG	−183	CGT ACGT GGC	Yamaguchi-Shinozaki et al. (1989)
CDeT27-45[a]	−213	TTG CACC GT	−196	GGC ACGT ATG	Michel et al. (1993)
Em	−69	ACG CACC GC	−95	CAC ACGT GCC	Litts et al. (1991)
HVA1	−79	GAG CACC GC	−99	CCT ACGT GGC	Straub et al. (1994)
Rab16B	−159	GCT CACC CA	−212	TAC ACGT CCC	Yamaguchi-Shinozaki et al. (1989)
Rab16C	−129	GCT CACC CC	−113	TAC ACGT ACC	Yamaguchi-Shinozaki et al. (1989)
	−150	ACG CACC AG	−182	CAC ACGT CCT	
	−207	CGT CACC CT	−234	CAT ACGT GGC	
LE25[b]	−93	ACT CACC AC	−130	AAA ACGT GTC	Cohen and Bray (1992)
Atrab18[c]	−340	CAG CACC CT	−408	ATT ACGT GTG	Lång and Palva (1992)

[a] *Craterostigma desiccation tolerance 27–45.*
[b] *Lycopersicon esculentum 25.*
[c] *Arabidopsis thaliana Rab18.*

upstream element, which includes ABRE1 and ABRE2, to confer any significant ABA induction might be due to either the alternation of the distance between ABRE2 and the TATA box when compared to the functional construct CX2 or the absence of a coupling sequence present in the downstream element. To exclude the first possibility, a 140-bp λ DNA fragment was inserted between ABRE2 and the minimal promoter so that the distance between ABRE2 and the TATA box in this construct (CX2E1::λ) would be similar to that in construct CX2. Clearly, the addition of this λ DNA fragment had no effect on ABA induction (compare CX2E1 to CX2E1::λ, Figure 6). Because we have shown that in this upstream element only ABRE2 is involved in ABA induction of *HVA22* (Figure 2), it appears that a coupling element (CE2) located in the internal −220 to −101 region is required to interact with ABRE2 in the upstream element to confer an ABA response similar to that described previously for ABA response complex 1. We designated this 176-bp fragment ABA response complex 2.

DISCUSSION

ABA regulates the expression of a variety of genes involved in seed development and a plant's response to environmental stresses. Barley *HVA22* is one of these genes, and its expression is tightly regulated by both stress and ABA. This single-copy gene is ubiquitous in all of the cereal species tested so far. We have determined with the particle bombardment technique that the effect of ABA on *HVA22* expression is most likely on its transcription because in a dosage–response experiment, the level of *HVA22* mRNA is well correlated with the level of *GUS* expression driven by the *HVA22* promoter (Shen et al., 1993). To define the *cis*-acting elements involved in high-level gene expression in response to ABA, we have performed a systematic analysis of the *HVA22* promoter. Three conclusions can be drawn from this study. First, not all G-box–like sequences are involved in the response to ABA. As we discussed previously, a particular G-box (ABRE1) is not necessary for the ABA response of the *HVA22* promoter. Similarly, we have reported that the ACGT-core–containing ABRE1 in another ABA-responsive gene, *HVA1*, is not involved in ABA responsiveness of that gene (Straub et al., 1994). Second, even when a G-box–like sequence is shown to be necessary for ABA response in the barley *HVA22* gene, it alone is not sufficient, and a minimal ABA response complex in this promoter is composed of a novel element and a G-box. Third, at least two ABA response complexes are present in the *HVA22* promoter; these two complexes contribute incrementally to the expression level of the gene in response to ABA. As summarized in Figure 7A, the first one, that is, the TATA-box–proximal ABA response complex 1, is a 49-bp fragment containing ABRE3 and CE1, and the second one, TATA-box–distal ABA response complex 2,

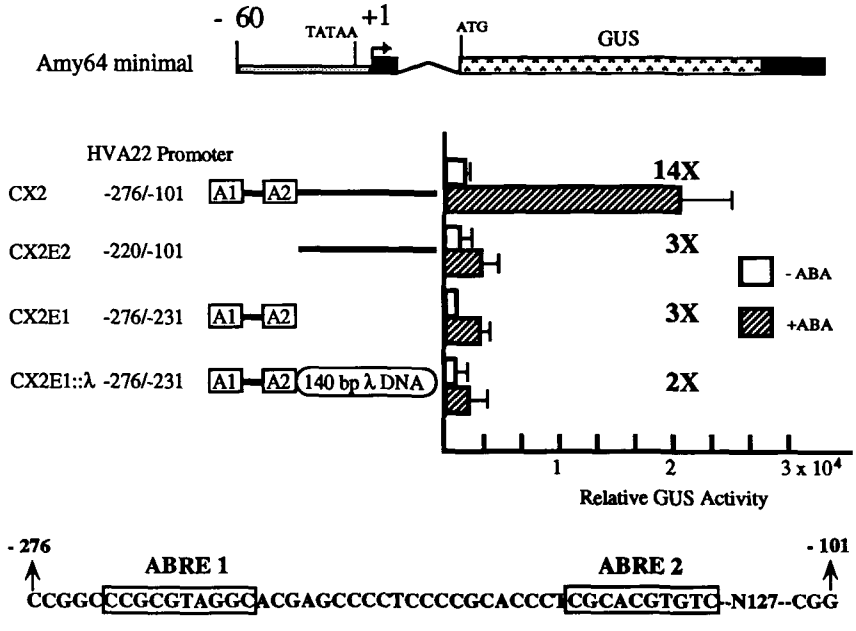

Figure 6. Another ABA Response Complex Present in the *HVA22* Promoter.

The 176-bp (−276 to −101) *HVA22* promoter, shown at the bottom, was fused to the *Amy64* minimal promoter (stippled rectangle) as described in Figure 4. The numbering of this *HVA22* promoter sequence is relative to the transcription start site. N127 indicates the distance between the two stretches of the sequence in base pairs. Symbols are as given in the legend to Figure 2. Relative GUS activity of each construct, calculated as described in Methods, is the mean of four replicas. Error bars indicate the standard error of each set of replicas. X indicates fold increase.

consists of ABRE2 and CE2, an undefined *cis*-acting sequence located between −220 and −101.

The necessity of both ABRE3 and CE1 for ABA responsiveness of ABA response complex 1 is clearly demonstrated in Figures 5A and 5B; mutation of either element almost completely abolished ABA induction from the gain-of-function construct 1C+. Furthermore, a 20-bp element containing ABRE3 and the 10-bp sequence immediately upstream of ABRE3 failed to confer ABA responsiveness to the *Amy64* minimal promoter used in the gain-of-function construct 1C+ (data not shown). ABRE3 is basically a G-box with the consensus ACGT core (for review, see Foster et al., 1994), whereas CE1 is a novel element.

CE1-like elements are present in all ABA-regulated genes that we have checked; some of these are listed in Table 1. It is intriguing that in most of these genes, the distances between the potential coupling elements and the G-box sequences are very similar to that in *HVA22*. However, it remains to be determined whether they are indeed the coupling sequences in those genes.

The definition of the second ABA response complex serves to elucidate the result from our linker-scan analyses of the native *HVA22* promoter in which we have shown that to attain a high level of ABA induction from a native *HVA22* promoter, it is necessary to have both ABRE2 and ABRE3 sequences (Figures 2 and 3). The mutation studies suggest that these two G-box sequences contribute incrementally to the expression level of the gene in response to ABA (Figure 3). Sequence replacements of both of these elements essentially abolish transcription from this promoter. Our gain-of-function study with the −276 to −101 promoter sequence (Figure 6) clearly demonstrates that there is another ABA response complex in the *HVA22* promoter. Similar to the case of ABA response complex 1, the presence of the yet-undefined coupling element located between −230 and −101 is required for interaction with the upstream ABRE2 sequence to confer a high level of ABA induction.

The "coupling model" described previously has at least partially resolved the puzzle for the involvement of the G-box in responding to a variety of different environmental and physiological cues. Similar to the observation that ABA response relies on the interaction of a G-box (ABRE3, GCCACGTACA, or ABRE2, CGCACGTGTC) with a coupling element (CE1, TGCCACCGG, or CE2), as represented in Figure 7B, the presence of both a G-box (box II, TCCACGTGGC, or box III, TGTACGTGGA) and another element (box I, GTCCCTCCAACCTAACC, or box IV, CTTCACTTGATGTATC) are necessary for the UV-light response of the chalcone synthase (*chs*) promoter (Schulze-Lefert et al., 1989). Specific point mutations within either box II or box I result in a dramatic reduction of light-induced gene expression (Block et al., 1990). Similarly, Donald and Cashmore (1990) have reported that a mutation in either the G-box (CTTCCACGTGGC) or I-box (I-1, AACGATAAGATT, and I-2, AGCCGATAAGGG) dramatically reduces the expression from the light-responsive Arabidopsis small subunit of ribulose bisphosphate carboxylase gene, *rbcS-1A*. In the case of the *chs*

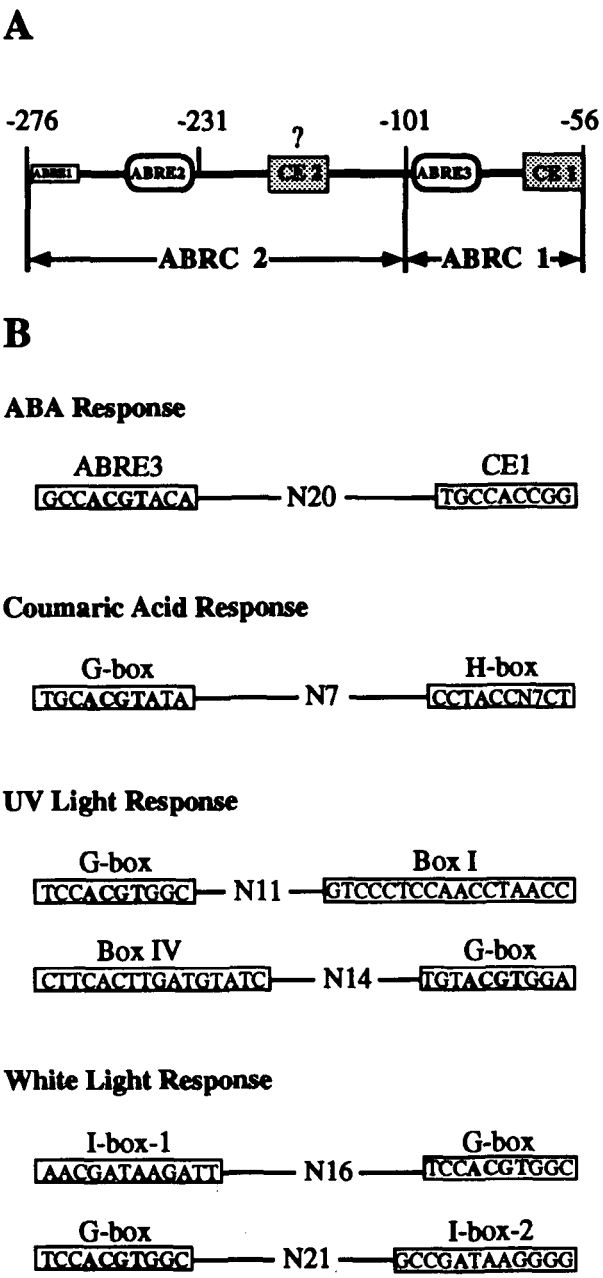

Figure 7. Coupling of a G-Box–like Sequence with Another *cis*-Acting Element in Determining Signal-Specific Responses.

(A) Two ABA response complexes (ABRC) are present in the *HVA22* promoter. The numbering is relative to the transcription start site. (?) indicates that CE2 is not defined.

(B) Schematic model of signal-specific complexes. The ACGT cores in the G-box sequences are in boldface letters. The distance between the G-box and the coupling sequence is indicated by the number (N) of nucleotides. The coumaric acid response complex is adopted from Loake et al. (1992), UV-light response complexes are from Block et al. (1990), and white-light response complexes are from Donald and Cashmore (1990).

gene, the combination of H-box (CCTACC-N_7-CT) and G-box (CACGTG) *cis*-acting elements is necessary for the response of this promoter to the phenylpropanoid pathway intermediate p-coumaric acid (Loake et al., 1992). Although the G-box sequences in these genes are similar, the elements interacting with them, as shown in Figure 7B, are different in the complexes involved in the response to ABA (this work), coumaric acid (Loake et al., 1992), UV light (Schulze-Lefert et al., 1989), and white light (Donald et al., 1990). Therefore, it appears that the signal response specificity is at least partially determined by the coupling elements (Figure 7B).

The flanking sequence around the ACGT core may also participate in determining the signal response specificity by differentiating the interactions with various ABRE or G-box binding proteins. These types of proteins have been identified in several plant species, including tobacco (TGACG sequence-specific binding proteins [TGA1a and TGA1b] and transcription activator factor-1 [TAF-1]), parsley (common plant regulatory factors [CPRF1, CPRF2, and CPRF3]), wheat (histone DNA binding proteins [HBP-1a and HBP-1b] and the Em binding protein [EmBP-1]), and maize (the octopine synthase enhancer binding factor [OCSBF-1]) (for review, see Foster et al., 1994). It has been shown by in vitro binding assays that the two bases flanking the CACGTG core affect the specificity of protein binding because these flanking sequences determine whether oligonucleotides possessing the same core have "type A" or "type B" binding activities in a cauliflower nuclear extract (Williams et al., 1992). However, this cannot completely account for the signal response specificity. For example, type A binding activity is obtained with elements involved in both ABA response (Em1a, GACACGTGGC of the wheat *Em* gene; Guiltinan et al., 1990) and light response (e.g., T/CCACGTGGC of *rbcS* genes; Williams et al., 1992). On the other hand, in the ABA-responsive genes, the sequences flanking the ACGT core vary dramatically from one G-box to another (Table 1). Hence, the flanking sequence cannot be the only determinant of signal response specificity. Rather, it may also rely on the interaction of two *cis*-acting elements as described in our model.

The presence of more than one signal response complex is not unique to the ABA-responsive *HVA22* gene. In the promoter of the parsley *chs* gene, two functionally independent "light-responsive *cis* units" apparently regulate the expression of the *chs* gene (Schulze-Lefert et al., 1989; Figure 7B). Similar to what we observed with the *HVA22* promoter in regulating ABA response (Figures 4 and 6), the TATA-proximal unit of the *chs* promoter is stronger in regulating light-induced gene expression (Schulze-Lefert et al., 1989). Interestingly, the sequences of box I and box IV, which couple with the ACGT-core–containing box II and box III, respectively, are dramatically different, yet both units can independently confer light responsiveness, although to a different extent. In another system, Liu et al. (1994) have shown that in the promoter of the soybean auxin-inducible *GH3* gene, three elements can independently confer auxin responsiveness to a minimal 35S (−46) promoter as a single-copy sequence. One of the sequences is D1, and it is located between −183 and −159; another is D4, and it resides at −142 to −111. The third completely different ACGT-core–containing sequence located between −249 and −203 (GGGAGAACTTTTGCTGACGT-GGCGACACATCTGGACCCACATGTCGG) can also function as an auxin response sequence when four tandem copies of the sequence are fused to the 35S minimal promoter (Liu et al., 1994).

The presence of two ABA response complexes might facilitate the regulation of the transcription strength of an ABA-regulated gene. In the *HVA22* gene, it appears that ABA response complex 1 and ABA response complex 2 could simultaneously interact with the basal transcription machinery in the native *HVA22* promoter. When ABRE2 is mutated, ABA response complex 2 is impaired in ABA responsiveness so that only ABA response complex 1 regulates the ABA response and consequently results in the decrease in the level of ABA induction from the mutated *HVA22* promoter. Probably because ABA response complex 1 is closer to the TATA-box or the complex is more stably associated with the basal transcription machinery, it is able to confer a higher level of ABA induction than ABA response complex 2. For the same reason, the mutation of ABRE3 has a greater negative effect on the ABA response than the ABRE2 mutation.

Proteins interacting with G-box sequences have been characterized (Guiltinan et al., 1990; Oeda et al., 1991; Armstrong et al., 1992; Schindler et al., 1992b). All of the proteins contain a basic region with an adjacent leucine zipper, that is, bZIP proteins (Landschulz et al., 1988). Among these, EmBP-1 binds to the ACGT-core–containing ABRE sequence of the ABA-responsive wheat *Em* gene (Guiltinan et al., 1990). Some of the plant bZIP proteins can form heterodimers and homodimers (Armstrong et al., 1992; Schindler et al., 1992a). In addition, VP1 has been shown to be a transcription factor (McCarty et al., 1991). The presence of VP1 is essential for the expression of the ABA-responsive *Em* gene because its mRNA was not detected in the *vp1* mutant. Furthermore, overexpression of the VP1 protein induces transcription from the *Em* promoter in maize cells. The overexpression of VP1 and the addition of ABA synergistically induce expression of the ABA-responsive *Em* promoter (McCarty et al., 1991). The VP1 protein does not appear to have a DNA binding domain or dimerization motifs (McCarty et al., 1991). Therefore, it is unlikely that VP1 would directly interact with ABRE or CE1. Rather, it may mediate protein–protein interactions. It is conceivable that EmBP and EmBP-like proteins might bind to ABRE3 and that another protein interacts with CE1. VP1 could then mediate the interaction of these proteins with factors associated with the TATA-box binding protein, RNA polymerase II, and other basal transcription factors (Comai et al., 1992). We have shown that ABA response complex 1 is apparently able to drive stronger transcription from the minimal promoter than from ABA response complex 2 (Figures 4 and 6). The mechanism underlying this observation might be due to the differences in binding affinities of transcription factors interacting with the ABA response

and/or coupling elements. Additional studies are under way to investigate the interactions between the G-box and the coupling elements.

METHODS

Chemicals

Oligonucleotides were synthesized in the Department of Biology at Washington University and by Integrated DNA Technologies Inc. (Coralville, IA). Luciferin was purchased from Analytic Luminescence Laboratory (San Diego, CA). Restriction enzymes were obtained from Promega (Madison, WI) and New England Biolabs (Beverly, MA). All other chemicals were purchased from Sigma.

Particle Bombardment and Enzyme Assays

Seed of barley (*Hordeum vulgare*) cultivar Himalaya (1985 and 1988 harvests; Department of Agronomy and Soils, Washington State University, Pullman, WA) were used throughout this study. Embryoless half-seed preparation, DNA construct bombardment, homogenization of the bombarded seed, and β-glucuronidase (GUS) and luciferase assays were conducted essentially as described previously (Shen et al., 1993). Each test construct containing the *GUS* reporter gene was mixed with the internal control construct pAHC18 (maize ubiquitin promoter/luciferase construct; Bruce and Quail, 1990) in 1:1 molar ratio. Half seeds, after being imbibed in 20 mM $CaCl_2$, 20 mM sodium succinate, pH 5.0, for 3 days, bombarded with the DNA mixture, and incubated with or without 2×10^{-5} M abscisic acid (ABA) for 24 hr, were ground with a chilled mortar and pestle in 0.8 mL of grinding buffer according to Lanahan et al. (1992) and Shen et al. (1993). After centrifugation in a microcentrifuge for 15 min, 100 μL of the supernatant was used for the luciferase assay. For GUS assays, 50 μL of the extract was mixed with 200 μL of GUS assay buffer (Lanahan et al., 1992; Shen et al., 1993) and incubated at 37°C for 20 hr. Fifty microliters of the GUS assay mixture was then diluted into 2 mL of 0.2 M Na_2CO_3 and measured in a Sequoia-Turner fluorometer (model 450; Fisher, Pittsburgh, PA) with 1 μM 4-methylumbelliferone giving a reading of 1000 units. The normalized GUS activity represents the total number of fluorescent units in 20 hr from an aliquot of extract that contained 2,000,000 relative light units of luciferase activity. Statistical analyses were conducted with the independent Student's *t* test.

DNA Construct Preparation

The KpnI-SalI fragment from PBgII/IGU (Shen et al., 1993), containing the *HVA22* sequence between the BglI site (−282) and SalI site (+46), was cloned into the corresponding sites of a modified pBluescribe vector to generate the progenitor XP1 or cloned into those of pBluescript SK− (Stratagene) to give another progenitor, XP2. After introducing XP1 and XP2 into an *Escherichia coli dut*$^-$, *ung*$^-$ strain (CJ236), single-stranded DNA was prepared. The sequence of the single-stranded DNA template generated from XP1 is complementary to the transcribed strand, whereas that from XP2 is the same as the transcribed strand. The oligonucleotides (listed in Table 2) that included a 6- to 10-bp mutation were hybridized to the single-stranded DNA templates obtained from XP1 (oligonucleotide OG06) or XP2 (OG01 through OG05). In vitro DNA synthesis, ligation, and transformation were conducted as described previously (Kunkel et al., 1987) to generate constructs X1 through X6. The mutated *HVA22* promoters were sequenced to ensure that no unwanted mutation was introduced.

For constructs LS-01 through LS-06, the mutated *HVA22* sequence (KpnI-SalI fragment) was isolated and fused to the corresponding sites of the construct Ps/bIGU, which contains the *HVA22* upstream

Table 2. Oligonucleotides Used in Making Promoter Constructs

Oligonucleotide	Sequence[a]	For Constructs
OG01	5′-CGGGGAGGGGCTCGTtgaattcattGCCGGGCCCGGTACC	LS-01
OG02	5′-GTGCGAGGGTGCGGGcGaattcgtaGCCTACGCGGGCCGG	LS-02
OG03	5′-CGCCGACACGTGCGAaGaattctaaAGGGGCTCGTGCCTA	LS-03
OG04	5′-GCGTGGCGGCACGCCtggAattctacGGGTGCGGGGAGGG	LS-04
OG05	5′-GTAGTGCTCCGCGTGtaGaattctaGACACGTGCGAGGGT	LS-05
OG06	5′-CCGGGTGCTTGGCGGCgaattcgcGGGCAGCCGGCATCC	LS-06
OG07	5′-ATTCGAGCTCGGTACtgaatTctatGCCACGTACACGCCA	LS-07
OG08	5′-GGTACCCGGCTGCCCatgAatTcgcCGCCAAGCACCCGGT	LS-08
OG09	5′-TGCCCGCCACGTACAgaattctagtCCGGTGCCATTGCCA	LS-09
OG10	5′-GTACACGCCAAGCACgaatTctacgTGCCACCGGGGATCC	LS-10
OG11	5′-AGCACCCGGTGCCATgtagAattctGATCCTCTAGGATCC	LS-11
OG12	5′-ATTCGAGCTCGGTACaattgTGCCCGCCACGTACA	LS-12
OG13	5′-AGCTCGGTACCCGGCaattgGCCACGTACACGCCA	LS-13
OG14	5′-GGTACCCGGCTGCCCaCaACGTACACGCCAAGCAC	LS-14
OG15	5′-CCGGCTGCCCGCCACGTttgCGCCAAGCACCCGGT	LS-15
OG16	5′-GTACACGCCAAGCACaattgGCCATTGCCACCGGG	LS-16
OG17	5′-CGCCAAGCACCCGGTttacaTGCCACCGGGGATCC	LS-17
OG18	5′-AGCACCCGGTGCCATcattgCCGGGGATCCTCTAG	LS-18
OG19	5′-CCGGTGCCATTGCCAGATCTgatcCTCTAGGATCC	LS-19

[a] The mutated sequences are shown in lowercase letters, and the EcoRI site, which was introduced for quick screening purposes, is underlined.

promoter (SalI-BglI) sequence, *HVA22* intron1–exon2–intron2, the *GUS* coding region, and the *HVA22 3′* region. During these cloning processes, the original BglI site in the *HVA22* promoter was destroyed, and a 17-bp insertion was introduced at this site. This insertion, however, had no effect on promoter activity at both the absolute level of GUS activity and ABA fold induction (data not shown). Insertion of the wild-type KpnI-SalI promoter fragment into the corresponding sites of Ps/bIGU generated LS-P. To prepare double mutants, uridine-containing single-stranded templates were prepared from two constructs, X1 (mutated ABA response element ABRE1) and X6 (mutated ABRE3). Double mutation constructs were then obtained with these templates by using the method of Kunkel et al. (1987) and with oligonucleotides OG04 (mutated ABRE2) and OG06 (mutated ABRE3). After sequencing for confirmation, the mutated sequences were ligated into Ps/bIGU to give the double mutants of the *HVA22* promoter, A1/A2 and A2/A3.

To prepare constructs for the gain-of-function study, a fragment containing the truncated (−60) promoter of the *Amy64* gene, which encodes a high-pI barley α-amylase (Khursheed and Rogers, 1988), and its 5′ untranslated region (downstream to +57 relative to the transcription start site) was ligated to a construct (IGU) containing *HVA22* intron1–exon2–intron2, the *GUS* coding region, and the *HVA22 3′* region, generating the construct MP64. One, two, three, or four copies of the 49-bp BsrFI-BsrFI fragment containing ABRE3 were cloned separately into the compatible XmaI site of MP64 to generate 1C+ (+ means the insert orientation is the same as in the *HVA22* promoter), 2C+, 3C+, and 4C+, respectively. One copy of the same 49-bp promoter sequence was inserted in the opposite orientation to generate 1C−. The intronless construct is similar to 1C+, except that the intron1–exon2–intron2 fragment was not included.

To prepare the constructs for the ABA response complex 2 analysis, the KpnI-BsrFI fragment from LS-P, containing −276 to −101 of the *HVA22* promoter, was cloned into KpnI- and XmaI-cut MP64 to give CX2. The upstream (−276 to −231) and downstream (−220 to −101) elements of this 176-bp fragment were cloned separately by inserting the EcoRI-EcoRI fragment of construct X6 into EcoRI-cut MP64 to generate constructs CX2E1 and the EcoRI-BsrFI fragment of X2 into EcoRI- and XmaI-cut MP64 to give CX2E2, respectively. In the construct CX2E1::λ, a 140-bp λ DNA fragment was inserted between ABRE2 and the minimal promoter so that the distance between ABRE2 and the TATA-box in this construct was similar to that in construct CX2. The uridine-containing single-stranded DNA template was prepared from construct 1C+ to generate constructs LS-07 through LS-19 in which the 49-bp *HVA22* promoter sequence was mutated at 10- or 5-bp intervals. The oligonucleotides used to generate the constructs used in this work are listed in Table 2.

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