Modular Nature of Abscisic Acid (ABA) Response Complexes: Composite Promoter Units That Are Necessary and Sufficient for ABA Induction of Gene Expression in Barley

Qingxi Shen, Pengnian Zhang, and Tuan-Hua David Ho

Plant Biology Program, Department of Biology, Division of Biology and Biomedical Sciences, Washington University, St. Louis, Missouri 63130

The modular nature of the abscisic acid response complex (ABRC), the promoter unit necessary and sufficient for abscisic acid (ABA) induction of gene expression in barley, is defined in this study. We investigated ABA induction of a barley late embryogenesis abundant (Lea) gene, HVA1, and found that the ABRC of this gene consists of a 10-bp box with an ACGT core (ACGT-box) and the 11 bp directly upstream, named coupling element 3 (CE3). Only one copy of this ABRC is sufficient to confer ABA induction when linked to a minimal promoter. Because we previously reported another ABRC in the barley HVA22 gene, which consists of an ACGT-box with a distal coupling element (CE1), exchange experiments were conducted to study the interaction among modular elements in these ABRCs. We show that ACGT-boxes in these ABRCs are interchangeable, indicating that an ACGT-box can interact with either a distal or a proximal coupling element to confer ABA response. However, the two coupling elements are not fully exchangeable. Although CE3 can function either proximal or distal to the ACGT-box, CE1 is only functional at the distal position. The presence of both the distal and the proximal coupling elements has a synergistic effect on the absolute level of expression as well as on ABA induction. These ABRCs function in both seed and vegetative tissues. In seeds, ABA induction of the ABRC containing the proximal CE3, but not the ABRC with the distal CE1, is enhanced in the presence of the transcription regulator Viviparous1, indicating that these two ABRCs are mediated by different ABA signal transduction pathways.

INTRODUCTION

Abscisic acid (ABA) is an important hormone mediating seed development and plant stress responses. The basis of ABA action in regulating these processes has been pursued for more than two decades yet still remains unclear. The response to ABA does not always require gene expression, as exemplified in the stomatal closure controlled mainly by ABA-regulated ion fluxes (reviewed in Hetherington and Quatrano, 1991). However, it has been found that ABA exerts some of its effects by altering the transcription level of genes. Examples of ABA-induced genes include Lea (late embryogenesis abundant) and Rab (response to ABA) from several plant species (reviewed in Mundy and Chua, 1988), Craterostigma plantagineum genes encoding aldose reductase and cytosolic glyceraldehyde-3-phosphate dehydrogenase (Bartels et al., 1994; Velasco et al., 1994), a wheat gene encoding L-isoaspartyl protein methyltransferase (Mudgett and Clarke, 1994), and a duckweed peroxidase gene (Chaloupkova and Smart, 1994). Hence, a fundamental understanding of the mechanism of ABA in regulating gene expression is essential for elucidating the mode of ABA action in many physiological processes.

Approaches adopted to fulfill this goal include the localization and characterization of the primary ABA receptor sites (Alaian et al., 1994; Anderson et al., 1994; Gilroy and Jones, 1994; Schwartz et al., 1994) and the study of ABA response mutants, such as maize Viviparous1 (VP1) and Arabidopsis ABA-insensitive (ABI1) (Leung et al., 1994; Meyer et al., 1994). It has been shown that VP1 and ABI1 gene products, a transcription factor (McCarty et al., 1991) and a protein phosphatase (Leung et al., 1994; Meyer et al., 1994), respectively, are components of an ABA signal transduction pathway(s). Identification of the cis- and trans-acting elements involved in the ABA response is another approach to complement the genetic analysis. ABA-responsive 5' upstream sequences and proteins binding to these regions have been identified in wheat (Marcotte et al., 1989) and rice (Mundy et al., 1990). Furthermore, Skriver et al. (1991) have demonstrated that six copies of the sequence GTACGTGGCGC conferred sixfold ABA induction to a cauliflower mosaic virus 35S minimal promoter. This sequence and its homologs have been designated as ABA response elements (ABREs; Guiltinan et al., 1990; Skriver et al., 1991; Shen et al., 1993). It has been pointed out by Michel et al. (1993) that both the defined and putative ABREs contain

\footnote{To whom correspondence should be addressed.}
Figure 1. An ACGT-Box and a Coupling Element Are Essential for the ABA Response of the HVA7 Gene Promoter.
(A) A linker-scanning study of the 68-bp promoter fragment containing an ACGT-box. A schematic diagram of the test construct is shown at the top; 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 (box with vertical lines) was from the HVA22 SphI-SphI genomic fragment, including the polyadenylation sequence (AATAAA). The minimal promoter (open box) from the Amy64 gene (extending from positions -60 to +57) is included to provide the TATA-box sequence. These five components form the progenitor construct MP64. A 68-bp seg-
an ACGT core. Interestingly, similar sequences, namely, G-boxes, also contain an ACGT core, and they are 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), anaerobiosis (McKendree and Feri, 1992), auxin (Liu et al., 1994), jasmonic acid (Mason et al., 1993), and salicylic acid (Qin et al., 1994). Because of this similar structure between an ABRE and a G-box, we collectively designated them as ACGT-boxes for the ease of presentation. Hence, an interesting question remains unanswered: What determines the signal response specificity of promoters containing ACGT-boxes?

It has been suggested that sequences flanking the ACGT core may be involved in determining the signal response specificity of a promoter. This suggestion is based on the observation that the binding patterns to the nuclear extract are determined by sequences flanking an ACGT core in gel mobility shift assays (Williams et al., 1992). Furthermore, different cloned ACGT binding basic leucine zipper (bZIP) proteins interact with G-box sequences with different affinities (Armstrong et al., 1992; Schindler et al., 1992; Izawa et al., 1993; Foster et al., 1994). Therefore, differential regulation could be mediated by the associations of different G-box binding proteins with different types of G-boxes.

However, our recent study with the barley ABA-responsive HVA22 promoter suggests that a coupling element is involved in the determination of the ABA response specificity. In the 49-bp HVA22 promoter fragment, mutation of either the ACGT-box, A3 (previously designated ABRE3), or the coupling element, CE1, located 20 bp downstream from A3, essentially abolishes the ABA response of the promoter (Shen and Ho, 1995). Based on this observation, we have designated the A3 and CE1 elements in the 49-bp HVA22 promoter fragment as ABA response complex 1 (ABRC1).

Is this the only promoter complex that mediates ABA response or are other types of complexes also involved? Recent evidence suggests that it is likely that more than one pathway mediates ABA response. For instance, VP1, a transcription factor in maize, has been shown to enhance the expression of many ABA-inducible genes, such as the wheat Em gene; it does not appear to be required for the ABA-mediated regulation of maize Catalase 1 (Cat 1) (Williamson and Scandalios, 1992) and Rab28 (Pia et al., 1991) genes. Therefore, it is possible that different transcription factors mediate the ABA-regulated expression of different genes, and accordingly, different cis-acting complexes are involved in the response to ABA.

To study this possibility, we characterized another barley ABA gene, HVA1 (Hong et al., 1988). Unlike HVA22, which encodes a potential regulatory protein, HVA1 belongs to group III Lea genes (Hong et al., 1988). In addition, the level of HVA1 gene expression is much higher than that of HVA22. We became interested in finding out whether the ABRC in the HVA1 gene is similar to or different from that in HVA22. The data presented in this study demonstrate that the ABRC in the HVA1 gene is composed of an ACGT-box, A2, and a coupling element (ACGCGTGTCCTC). However, this proximal coupling element is located immediately upstream of the ACGT-box and hence is different from the distal CE1 in the HVA22 promoter in terms of both its location and sequence. Experiments with exchanges between the two ACGT-boxes and coupling elements demonstrated that an ACGT-box could interact with either a distal or a proximal coupling element to confer ABA response. However, the distal and proximal coupling elements are not fully exchangeable. Taken together, these data indicate that the HVA1 and HVA22 genes contain distinct ABRCs. Interestingly, expression of the maize VP1 transcription factor in barley aleurone layers activates the ABRC in the HVA1 gene but not in the HVA22 gene. In addition, the presence of VP1 and ABA treatment has a synergistic effect on the ABRC of the HVA1 gene but not on that of HVA22.

RESULTS

The HVA1 Promoter Contains an ABRC Different from Those in HVA22

We have shown previously that at least two ACGT-boxes (A2 and A3) in the barley HVA1 promoter are necessary for ABA induction (Straub et al., 1994). A 68-bp fragment (from positions −134 to −67 relative to the transcription start site) containing A2 was fused to a minimal promoter (Amy64) and the coding sequence of the β-glucuronidase (GUS) reporter gene in construct MP64 in an orientation shown in Figure 1. Clearly, this short fragment was able to confer a high level of

Figure 1. (continued).

ment of the HVA1 promoter sequence (from positions −134 to −67) was fused 3' to the minimal promoter. This 68-bp region is subdivided into six fragments, designated as fragments I, II, III, IV, V, and VI, and their sequences were replaced individually. The lowercase letters in the promoter sequence represent the bases that are mutated, and the dashed lines indicate the sequence identical to the wild type (WT). To the right are the levels of GUS activities expressed from each construct. The white box represents the relative GUS activity of samples from aleurone layers incubated in the absence of ABA, and the filled box represents those from aleurone layers incubated in the presence of 2 × 10−5 M ABA. Here and in Figures 2 to 7, the relative GUS activity of each construct, calculated as described in Methods, is the mean of four replicates. The error bar indicates the standard error of each set of replicates. X indicates fold induction.

B Detailed mutation studies indicate that CE3 is a novel coupling element. The HVA1 promoter segment containing fragments III (CE3), IV (A2), and V, as well as mutated (regions surrounded by rectangular boxes) fragments (I, II, and VI), is fused to the minimal promoter sequence, as described above. All other symbols and experimental conditions are the same as given in (A).
ABA induction (construct C1). To dissect the region important for ABA response, this promoter fragment was subdivided into six segments, designated I, II, III, IV(A2), V, and VI, which were mutated individually by the method of oligonucleotide-directed mutagenesis. Sequence replacement of segment I, II, or VI had little effect on the promoter activity (constructs C2, C3, and C7; Figure 1A). Mutating segment V, the 11 bp downstream of A2, had some negative effect on the absolute level of GUS expression and ABA induction, yet this mutant was still able to confer 21-fold induction (C7). In contrast, mutation of fragment IV (A2) almost completely abolished the ABA response of the promoter (construct C5), as did the substitution of the 12-bp sequence immediately upstream of A2, namely, segment III (construct C4). Therefore, the ABA response of this 68-bp promoter sequence is not only dependent on the presence of the ACGT-box (A2) but also on fragment III.

Because the individual mutations of fragment I, II, or VI have little effect on ABA response, we prepared a triple mutant to define further the sequence necessary and sufficient for an ABA response within the 68-bp HVA1 promoter fragment. As presented in Figure 1B, this triple mutant was still able to confer 15-fold ABA induction. Interestingly, both the middle part of HVA22 CE1 (Shen and Ho, 1995) and the 3' end of fragment III (this study) are rich in cytosine. We reasoned that if only the C-rich sequence in a coupling element was crucial for ABA response, then the 5' end of fragment III, which is quite different from the HVA22 CE1, might not be important for ABA response. In that case, fragment III would be another version of the CE1 sequence. To test this possibility, we prepared a series of substitution mutants of the 5' end of fragment III and studied their abilities to confer ABA response. Mutation of the first nucleotide in fragment III had little effect (C9). Replacement of the first three bases, however, reduced ABA induction from 15-fold to only threefold (C10). Similarly, when the 7-bp sequence at the 5' end was mutated, only twofold ABA induction was obtained. These data suggest that fragment III in the HVA1 gene is different from CE1 in the HVA22 promoter. Like CE1, however, fragment III could couple with an ACGT-box to confer a high level of ABA response. Hence, we designated fragment III in the HVA1 promoter as CE3.

A 22-bp HVA1 Promoter Sequence Is Sufficient to Confer an ABA Response

Data in Figures 1A and 1B suggest that the most critical elements in the HVA1 promoter are CE3 and A2. Thus, we fused this 22-bp fragment in a positive orientation (the same as in the native promoter) to a minimal promoter (Amy64) to study whether it is sufficient to support ABA induction. Unexpectedly, as shown in Figure 2, this 22-bp fragment was unable to confer an ABA response when it was fused immediately next to the minimal promoter (C13). The addition of fragment V, however, constructed a functional ABA response sequence that was able to confer 13-fold ABA induction (construct C12). Interestingly, when fragment V in construct C12 was replaced with a random sequence, the mutant was still able to confer a ninefold induction (Figure 2). Because nonfunctional C13 and functional C14 differed only in the distance between the HVA1 promoter fragment and the TATA-box in the minimal promoter, we wondered whether in C13 the 22-bp HVA1 sequence was placed too close to the TATA-box, thereby preventing proper interaction with the basal transcription machinery. When this 22-bp HVA1 sequence was placed 11 bp farther upstream with the insertion of either fragment V (C12) or a random sequence (C14), this short fragment was able to confer an ABA response. As more copies of the 22 bp were added, higher levels of GUS expression and ABA induction were obtained. Two copies (44 bp) of the CE3–A2 fragment were able to confer a 20-fold induction; three copies (66 bp) increased the absolute level of GUS activity more than three times (Figure 2). These data clearly indicate that the 22-bp fragment alone is indeed able to confer an ABA response. Similar results were obtained with at least two other minimal promoters, that is, those from a barley protease gene (EPB) and a rice actin gene (Q. Shen, P. Zhang, and T.-H.D. Ho, unpublished data). Hence, it appears that all information necessary and sufficient for ABA induction is present in the 22-bp fragment containing CE3 and A2. Because CE3 shares little homology with the CE1 element, we designated this functional 22-bp HVA1 promoter as ABRC3.

An ACGT-Box Can Interact with Either a Distal or a Proximal Coupling Element to Confer an ABA Response

It has been shown that to achieve a high level of ABA induction, the ACGT-box in the HVA22 promoter, that is, A3, has to interact with a distal element, CE1 (Shen and Ho, 1995), and that in HVA1, the ACGT-box, A2, needs to couple with the neighboring sequence, CE3 (Figure 1). Both A3 and A2, just like other ACGT-boxes, contain an ACGT core. In contrast, the two coupling elements, CE1 and CE3, in these two ABRCs are quite different. To test whether the two ACGT-boxes are equivalent, exchange experiments were conducted between ACGT-boxes in the 49-bp HVA22 ABRC1 and the 68-bp HVA1 ABRC3.

The sequences of these two complexes are shown in Figure 3A, and the ABA induction obtained from the wild-type and mutant promoter fragments is shown in Figure 3B. As reported previously (Shen and Ho, 1995) and redemonstrated here, A3 is indispensable for the ABA response of the HVA22 ABRC1. Mutation of A3 almost completely abolished the ABA response of the complex (C18). However, A2 sequence from the HVA1 gene could replace A3 in the context of the HVA22 ABRC1, giving a 38-fold induction (C19). As expected, the function of A2 in this promoter context is also dependent on the presence of the coupling element, CE1. When the CE1 element in the chimeric promoter was mutated, no ABA induction was obtained (C20), similar to the behavior of the HVA22 CE1 mutant (C21). Together, these data suggest that either ACGT-box (A2 or A3) could interact with the distal coupling element CE1 to confer an ABA response.
We reasoned that CE1 might not be necessary if the CE3 from *HVA1* were present because we have shown that the interaction of A2 and CE3 is necessary and sufficient for the ABA response in the *HVA1* native context. Indeed, the chimeric construct containing both CE3 and A3 (C22) or A2 (C23) in the CE1 mutation context still conferred an ABA response, leading to a 17- or 15-fold induction, respectively (Figure 3B). A similar exchange experiment was also performed in the context of the 68-bp *HVA1* promoter sequence. Similar to A2 (C1), A3 could interact with the proximal CE3 and conferred 24-fold ABA induction (C24) (Figure 3B). The requirement of CE3 for the function of this type of ABRC was further demonstrated in construct C25, in which the replacement of CE3 with fragment a from *HVA22* promoter abolished the ABA response. Therefore, the association of an ACGT-box with the proximal coupling element, CE3, can also comprise an ABRC.

**CE1 and CE3 Are Not Fully Interchangeable**

Because A3 and A2 are interchangeable in the context of both *HVA22* and *HVA1* promoters, we wanted to determine whether CE1 and CE3 could also substitute for each other. Hence, the 9-bp *HVA22* CE1 and the 12-bp *HVA1* CE3 elements were exchanged. As shown in Figure 4, CE3 could completely replace the function of CE1 in the *HVA22* promoter context in terms of the absolute level of GUS expression (C26). The somewhat lower ABA induction obtained from this construct was due to the higher background level (no ABA). Therefore, CE3 is able to interact with A3 at both a proximal location (C22; Figure 3) and a distal location (C26; Figure 4) to confer an ABA response. In contrast, CE1 could not function well when it was put at the place of CE3; only ninefold induction was obtained (C27; Figure 4), compared with the 52-fold induction from the wild type (C1; Figure 3). It should be noted that CE1 was able to interact with A2 to confer ABA response when it was at a distal location (C19; Figure 3). Therefore, it appears that the function of CE1 is position dependent.

Interestingly, the presence of both CE1 and CE3 appears to have a synergistic effect on the expression of the chimeric promoters. The expression level of C28, in which A2 was coupled with both CE3 and CE1 (Figure 4), was much higher than the sum of those from C19 and C23 (Figure 3). The synergistic effect was even greater in construct C29 containing CE3, A3, and CE1 (Figure 4); the levels of expression and ABA induction from this construct were much higher than those from C17 and C22 (Figure 3).

**Construction of Molecular Switches with Different Levels of ABA Induction and Transcription Strengths**

The delineation of ABRC1 and ABRC3 led us to conclude that the ACGT-boxes in the *HVA1* and *HVA22* promoter can confer
Figure 3. Exchange Study Demonstrating That an ACGT-Box Can Form a Functional ABRC with Either the Distal CE1 or the Proximal CE3.
Modular Nature of ABA Response Complexes

a high level of ABA response, provided that they are coupled with a distal or a proximal coupling element, namely, CE1 or CE3. At the same time, several recombinant DNA constructs were shown to be able to drive the expression of the GUS reporter gene at high levels. These ABA molecular switches are summarized in Figure 5. As reported previously, one copy of the 49-bp HVA22 promoter sequence containing ABRC1 is able to confer >30-fold ABA induction (Shen and Ho, 1995). Adding more copies of ABRC1 to the reporter construct led to an even higher level of ABA induction (Figure 5A). The 68-bp HVA1 promoter sequence containing ABRC3 turned out to be even stronger than the HVA22 ABRC1; one copy of this fragment led to 20-fold induction, with the absolute level of GUS activity twice as high as that obtained with the HVA22 ABRC1 (Figure 5B). Moreover, the presence of two coupling elements further enhanced the expression of the construct when they interacted with the ACGT-box from either HVA22 or HVA1 (Figures 5C and 5D). This is especially true for construct C29 (Figure 4), which contains the HVA22 ACGT-box (A3) and both the CE1 and CE3 coupling elements; one copy of this 61-bp promoter conferred 40-fold ABA induction, with the expression level as much as four times higher than that of the one-copy 49-bp HVA22 ABRC1 (Figures 5A and 5C).

ABA Molecular Switches Are Also Functional in Barley Vegetative Tissues

All of the data reported above were obtained with barley aleurone tissues. However, it is known that both HVA1 and HVA22

Figure 3. (continued).

(A) The 49-bp HVA22 promoter sequence containing ABRC1 and the 68-bp HVA1 promoter sequence containing ABRC3. The HVA22 promoter sequence is divided into five segments designated a, A3, c, d, and CE1; the first four segments are each 10 bp long, and the last one is 9 bp long. Similarly, the 68-bp HVA1 promoter is divided into six segments designated I, II, CE3, A2, V, and VI. The first three elements are each 12 bp long, A2 is 10 bp long, and segments V and VI are each 11 bp long.

(B) ABA induction and expression levels of recombinant ABRCs. The open regions in the promoter constructs are from the HVA22 promoter, and the black regions are from the HVA1 promoter. Rectangular boxes surrounding the A3 and CE1 elements indicate that the CE1 and A3 elements in those constructs are replaced with random sequences. The relative GUS activity of each construct, calculated as described in Methods, is the mean of four replicates. The error bar indicates the standard error of each set of replicates. X, fold induction. All other symbols are as given in Figure 1.
genes are also expressed in vegetative tissues (Hong et al., 1992; Q. Shen and T.-H.D. Ho, unpublished results). To determine whether these defined ABRCs also function in the vegetative tissues, we introduced constructs C17 with the 49-bp HVA22 promoter sequence containing ABRCl and C1 with the 68-bp HVA1 promoter sequence containing ABRC3 into 6-day-old barley leaf tissue. Data shown in Figure 6 demonstrate that both ABA switches were able to confer ABA induction in the vegetative tissue. As observed with the aleurone tissue, the HVA1 ABRC3 was more responsive to ABA than was the HVA22 ABRC1.

ABRC3, but Not ABRC1, Is Responsive to Transcription Factor VP1

The data presented above demonstrate that ABRC3 is different from ABRC1 in terms of its transcription strength and structure. However, what is the biological significance in having more than one ABRC? One possibility is that different ABRCs are mediated by different signal transduction pathways. This is based on the report that ABA-induced expression of certain genes, for instance, maize Rab28 (Pla et al., 1991), is VP1 independent, whereas ABA induction of other genes, such as the wheat Em gene (McCarty et al., 1991), is VP1 dependent. To test this hypothesis, we cobombarded the effector construct consisting of the maize VP1 coding sequence driven by a constitutive 35S promoter (McCarty et al., 1991) along with the reporter construct C1 (containing ABRC3) or C17 (containing ABRC1). McCarty et al. (1991) have shown that coexpression of VP1 in maize protoplasts enhances the wheat ABA-responsive Em promoter, and the presence of both VP1 and ABA has a synergistic effect. As shown in Figure 7 (right), a similar pattern of VP1 activation on ABRC3 of the barley ABA-responsive HVA1 gene was observed in our system. Coexpression of VP1 led to a fourfold induction of ABRC3, compared with the 14-fold induction by ABA. In the presence of both ABA and VP1, the induction increased to 31-fold, suggesting a synergistic effect of ABA and VP1 on ABRC3. In contrast, ABRC1 did not appear to respond to VP1 at all (Figure 7, left). In the absence of ABA, VP1 coexpression failed to activate ABRC1, giving no induction (onefold). The presence of VP1 and ABA gave a result (17-fold) similar to ABA treatment alone (15-fold). Therefore, VP1 appears to differentiate ABRC3 from ABRC1 in mediating ABA response.

Figure 5. List of DNA Molecular Switches Controlling the Expression of ABA-Inducible Promoters.

(A) The HVA22 complex consists of an ACGT-box and a distal CE1. The normalized GUS activity from the ABA-treated sample of the single-copy ABRC1 construct is taken as 100% in (A) to (D). Fold stands for fold induction, calculated as described in Figure 1A.

(B) The ABRC in the HVA1 promoter consists of an ACGT-box (A2) and the proximal CE3.

(C) and (D) The ternary ABRCs consist of two coupling elements and an ACGT-box.
Modular Nature of ABA Response Complexes

DISCUSSION

Analysis of the ABA-responsive gene HVA1 has led to the discovery of a novel type of ABA response complex, ABRC3, in which both the ACGT-box (A2) and the proximal coupling element CE3 are essential for the ABA response. The requirement of these two elements for ABA responsiveness is demonstrated not only in the loss-of-function studies (Figure 2B) but also in the gain-of-function studies (Figure 2). The 22-bp HVA1 promoter containing the ACGT-box and CE3 sufficiently confers an ABA response to a minimal promoter. This is reminiscent of ABRC1 in the HVA22 promoter, in which both an ACGT-box, A3, and a coupling element, CE1, are essential for ABA induction; mutation of either element almost completely abolishes ABA induction for this construct (Figure 3B; Shen et al., 1993). However, the sequences of these coupling elements, CE1 and CE3, and their locations relative to the ACGT-box appear to be quite different in these ABRCs.

Experiments designed to exchange components between the HVA1 and HVA22 promoters allowed us to define the modular nature of these ABRCs. Basically, ACGT-boxes in these two different ABA response complexes, ABRC1 and ABRC3, are interchangeable, suggesting that an ACGT-box can interact with either a distal element (CE1) or a proximal element (CE3) to confer an ABA response. In contrast, CE3 and CE1 are different because CE1 can only function at its native location, whereas CE3 can interact with an ACGT-box at either the proximal or the distal location. Interestingly, the presence of both CE1 and CE3 makes the ABRC much more responsive to ABA. These defined ABRCs function not only in barley aleurone but also in vegetative tissues. In seeds, the transcription regulator, VP1, enhances the expression of constructs containing ABRC3, regardless of whether ABA is present. However, ABRC1 is insensitive to VP1.

The definition of ABRCs in HVA1 and HVA22 genes suggests that the specific ABA response relies on combinatorial effects.

Figure 6. Both the 49-bp HVA22 Promoter Containing ABRC1 and the 68-bp HVA1 Promoter Containing ABRC3 Are Functional in Vegetative Tissue. The DNA constructs were bombarded into leaf tissue from 6-day-old greenhouse-grown barley plants and treated with or without 100 μM ABA in H2O at 24°C for 24 hr. The relative GUS activity of each construct, calculated as described in Methods, is the mean of four replicates. The error bar indicates the standard error of each set of replicates. X, fold induction. All other symbols are as given in Figure 1.

Figure 7. ABRC3, but Not ABRC1, Is Activated by the Maize VP1 Transcription Regulator. The 35S–Sh–VP1 construct containing the VP1 coding sequence driven by the 35S constitutive promoter was co-bombarded into barley aleurone layers along with the construct containing ABRC1 (C17) or ABRC3 (C1) at a 1:3 ratio (ABRC construct/VP1 construct). Similar results were obtained at a 1:0.2 ratio. Symbols below the bars indicate treatments with (+) or without (−) ABA and the VP1 effector construct. The relative GUS activity of each construct, calculated as described in Methods, is the mean of four replicates. The error bar indicates the standard error of each set of replicates. X, fold induction.
of two elements: an ACGT-box and a coupling element (this study; Shen et al., 1993). Our results agree with the observations of Rogers and Rogers (1992), who studied hormone response complexes in barley α-amylase gene promoters. They suggest that a specific sequence serving as a coupling element in a given promoter greatly affects where and when hormone response elements are able to regulate gene expression. In contrast, studies using other systems suggest that the ACGT-box itself constitutes sufficient information for a given signal response. For instance, a synthetic promoter containing six copies of an ACGT-box (GTACGTGGCGC) from the RabiA gene has been shown to confer an ABA response to a minimal promoter (Skriver et al., 1991). However, Vasil et al. (1995) have demonstrated that multimerization of ACGT-boxes may introduce cooperative interactions that might not be associated with a native promoter context. In their study, a tetramer of a 22- or 24-bp ACGT-box–containing sequence confers ABA responsiveness, regardless of whether it was derived from either the ABA-responsive Em gene or the light-responsive chalcone synthase gene promoter (Vasil et al., 1995). Similarly, a tetramer of a 39-bp ACGT-box containing the promoter sequence from the Agrobacterium octopine synthase gene responds to both salicylic acid and auxin (Zhang and Singh, 1994).

Because ACGT-boxes are involved in responses to a variety of different environmental and physiological cues, such as light (Donald and Cashmore, 1990), methyl jasmonate (Mason et al., 1993), and anaerobiosis (McKendree and Feri, 1992), it is unclear what determines the specificity in responding to this diverse group of signals. It has been suggested that the flanking sequences of the ACGT core may be the determinant of signal response specificity. The sequences flanking ACGT cores have been shown to affect the affinity for DNA binding factors (Williams et al., 1992). However, the flanking sequences of ACGT-boxes are quite different, even in the promoters responding to the same signal, as exemplified in A3 (GCCACGTACA) of ABRC1 and A2 (CCTACGTGGC) of ABRC3 (Figure 3A). Mutation studies further suggest that other ACGT core flanking sequences can confer an ABA response in the context of these two complexes (Q. Shen, P. Zhang, and T.-H.D. Ho, unpublished data). Therefore, whether the flanking sequences of an ACGT core are involved in the determination of specificity needs to be investigated further by using synthetic promoters containing a single copy of the ACGT-box, such as C1 and C17 reported in this study (Figure 3).

The definition of ABRCs in our studies suggests an alternative mechanism of response specificity determination. Even though the ACGT-boxes are similar, the coupling elements are different in complexes responding to ABA (this study; Shen and Ho, 1995), UV light (Schulz-LeFert et al., 1989; Block et al., 1990), white light (Donald and Cashmore, 1990), and p-coumaric acid (Loake et al., 1992). Hence, our data favor the model in which the interaction of an ACGT-box with a different coupling element determines the signal response specificity. Indeed, ABRC1 and ABRC3 respond only to ABA but not to coumaric acid, methyl jasmonate, or salicylic acid (Q. Shen, P. Zhang, and T.-H.D. Ho, unpublished data).

The presence of more than one type of ABRC may facilitate the regulation of ABA-responsive genes via different signal transduction pathways. There appears to be more than one ABA signal transduction pathway in a cell. Maize VP1 has been shown to be a transcription factor mediating seed development, including ABA responses in seed tissues (McCarty et al., 1991). Recently, it was reported that VP1 appears to enhance the expression of the wheat Em gene (Vasil et al., 1995). However, a VP1-independent pathway has been suggested to be involved in the expression of other ABA-responsive genes, such as maize Rab26 (Pia et al., 1991) and Cat1 (Williamson and Scandalios, 1992). Our study clearly demonstrates that ABRC3 can be affected by VP1, whereas ABRC1 appears to be VP1 insensitive. Based on these data, we suggest that the ABRC3 type of promoter complex is part of the VP1-dependent ABA response pathway and that the ABRC1 type of promoter complex is involved in the VP1-independent pathway. The barley ABRC3 shares some sequence and functional similarities with the Sph complex defined for ABA and VP1 regulation of the maize C1 promoter (Hattori et al., 1992). Fourteen base pairs in ABRC3 are identical with those in Sph, and both ABRC3 and Sph show synergism between VP1 and ABA. However, Sph in the maize C1 gene does not contain an ACGT core.

Transcription strength, ABA sensitivity, and tissue specificity may also be mediated by different ABRCs. As shown in Figure 5, the combination of different ACGT-boxes and coupling elements leads to the formation of ABRCs with different transcription strengths. In general, ABRC3 in the context of the HVA1 promoter has higher levels of expression than ABRC1 in the HVA22 promoter. However, ABRC1 and ABRC3 do not display tissue specificity; both of them function in aleurone tissue as well as in vegetative tissues (Figures 2 and 6). Given that barley HVA1 and HVA22 genes are expressed in both seeds and vegetative tissues, it is not expected that ABRC1 and ABRC3 carry any determinant for tissue-specific expression. A rice ABA-responsive gene, which is mainly expressed in vegetative tissues, has been reported by Yamaguchi-Shinozaki et al. (1989). It will be interesting to define the ABRC in this particular gene and compare it with the ABRCs we reported here.

The synthetic promoters presented in this study could be explored further for use in biotechnological applications. These molecular switches capable of conferring different levels of ABA induction could be used to drive the expression of genes that would enhance plant stress tolerance. For example, it has been shown that overexpression of a bacterial gene encoding mannitol 1-phosphate dehydrogenase could increase the ability of plants to tolerate high salinity (Tarczynski et al., 1993). However, this gene was driven by a constitutive 35S promoter, leading to the accumulation of mannitol even when the transgenic plants were not under stress. Because it has been reported that environmental stress, including salt, cold, and drought, results in an increase of the ABA level in a cell, substitution of the 35S promoter with one of our synthetic promoters
would put the bacterial gene under the control of an ABA-responsive switch, which would cause the gene to be expressed mainly in response to stress. The fact that the synthetic promoters also function in vegetative tissues has made it more plausible to apply these switches in a biotechnology project similar to the one described above.

**METHODS**

**Particle Bombardment and Transient Expression Assays**

The detailed procedures of transient expression studies with the barley (*Hordeum vulgare*) aleurone system and the particle bombardment technique have been published elsewhere (Lanahan et al., 1992; Shen et al., 1993). Briefly, the mixture (in a 1:1 molar ratio) of a test promoter–β-glucuronidase (GUS) reporter construct and a maize ubiquitin–luciferase internal control construct was bombarded into barley embryoless half seeds (four replicates per test construct). After incubation in the presence or absence of 20 μM abscisic acid (ABA) for 24 hr, the bombarded seeds in sets of four were homogenized in 800 μL of the grinding buffer (Shen et al., 1993). After centrifugation at 12,000g for 10 min at 4°C, 100 μL of the supernatant was assayed for luciferase activity. For the GUS assay, 50 μL of the supernatant was diluted into 200 μL of GUS assay buffer (Shen et al., 1993) and incubated at 37°C for 20 hr. Fifty microliters of the reaction mixture was then diluted into 2 mL of 0.2 M Na2CO3, and the resulting fluorescence was measured in a fluorometer (Sequoia-Turner model 450; UNIPATH, Mountain View, CA) in which 1 μM 5-methylumbelliferone gave 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.

Studies of ABA response in vegetative tissues were performed with 6-day-old barley plants grown in the greenhouse (14 hr of light/10 hr of dark at 20°C). Ten pieces of 1.5- to 2-cm-long leaf segments were bombarded with test constructs as well as the internal control luciferase construct mentioned earlier. Because the transformation efficiency of vegetative tissues is much lower than that of aleurone tissues, leaf segments from six individual shots were combined and divided into two portions and incubated with or without 10-4 M ABA in H2O for 48 hr. The treated vegetative tissues were processed the same as aleurone layers, and the relative GUS activity of each construct was the mean of four replicates (i.e., 24 shots for each construct).

**Preparations of the Test DNA Constructs**

The progenitor for all constructs reported in this paper is MP64, which was obtained by fusing the truncated (−60) promoter of the Amy64 gene. The detailed procedures used in making promoter constructs are described in Table 1.

### Table 1. Oligonucleotides Used in Making Promoter Constructs

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Construct</th>
</tr>
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<tbody>
<tr>
<td>a</td>
<td>5'-TTGAGCTCTGTCATcatcttagaGGGAGCGGCAACG-3'</td>
<td>C2</td>
</tr>
<tr>
<td>b</td>
<td>5'-ACCCGAAGGTACCGCcatcttagagtACGCTGTCTCTCCTC-3'</td>
<td>C3</td>
</tr>
<tr>
<td>c</td>
<td>5'-AAaGatcttagTCACCTACGTGCC-3'</td>
<td>C4</td>
</tr>
<tr>
<td>d</td>
<td>5'-GACACGCTGTCTCTCagaattacatGGCCATGTACAGA-3'</td>
<td>C5</td>
</tr>
<tr>
<td>e</td>
<td>5'-TCCCTCTCTACGTCatcAGAACCGCGCTACG-3'</td>
<td>C6</td>
</tr>
<tr>
<td>f</td>
<td>5'-TGGCGCCATAGTCatcAGAACCGCGCTACG-3'</td>
<td>C7</td>
</tr>
<tr>
<td>g</td>
<td>5'-TTCGAGCTCATcatcttagaGGGAGCGGCAACG-3'</td>
<td>C8</td>
</tr>
<tr>
<td>h</td>
<td>5'-GTTAACATCTAGTAaCCTGCTGCTCCTCCCTACG-3'</td>
<td>C9</td>
</tr>
<tr>
<td>i</td>
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<td>C10</td>
</tr>
<tr>
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<td>k</td>
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<td>l</td>
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<td>m</td>
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<td>u</td>
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<tr>
<td>z</td>
<td>5'-GCAACGCGCTGCTGCGCGCGACGCGCGCGC-3'</td>
<td>C27</td>
</tr>
</tbody>
</table>

*The mutated sequences are shown in lowercase letters, and restriction enzyme sites, which were introduced for quick screening of mutant, are underlined.*
gene (Khursheed and Rogers, 1988) and its 5’ untranslated region (downstream to position +457 relative to the transcription start site) to a construct containing HVA22 intron1-exon2-intron2, the GUS coding region, and the HVA22 3’ region (Shen and Ho, 1995). A 68-bp EcoRI-NruI HVA1 promoter fragment from the previously published construct LS-pA3 (Straub et al., 1994) was polished with the Klenow fragment of DNA polymerase I and ligated into the SmaI-digested MP64 construct, generating C1. The sequences of oligonucleotides used in preparing constructs mentioned below are listed in Table 1.

The 68-bp HVA7 promoter fragment in C1 was substituted at 10- to 12-bp intervals by using the method of oligonucleotide-directed mutagenesis, as described by Kunkei et al. (1997), to produce six mutants, C2, C3, C4, C5, C6, and C7. Two subsequent mutations of construct C7 with oligonucleotides b and g generated C8, which contains only CE3, A2, and fragment V. Mutation of 1 or 7 bp of CE3 with oligonucleotides h and j produced C9 and C11, respectively. C10 was prepared with oligonucleotide i, with C11 as the template. To prepare C12, two complementary oligonucleotides (k and l) were annealed and cloned into the EcoR1-Xbal-digested MP64. C13 was prepared as described for C12 but with oligonucleotides m and n. Mutation of the 11 bp sequence downstream from A3 in C12 with oligonucleotide o generated C14. C15 and C16 were prepared with two pairs of complementary oligonucleotides (p and q, and r and s, respectively) and SacI-EcoR1-digested C14. The preparation of constructs C17, C18, and C21 has been described by Shen and Ho (1995).

C19 and C20 were obtained by replacing the A3 sequence of the HVA22 gene with A2 of the HVA1 gene, using oligonucleotide t and the single-stranded DNA templates from construct C17 and C21, respectively (Shen and Ho, 1995). Similarly, C22 was prepared with C21 single-stranded DNA templates and oligonucleotide u. C23 was obtained by substituting the 10-bp HVA22 sequence upstream of the A2 in C20 with the 12-bp HVA1 sequence, using oligonucleotide v. Using oligonucleotide w, A2 in C1 was replaced with A3 of HVA22 to produce C24, from which C25 was constructed with oligonucleotide x. C26 was derived from the C17 template with oligonucleotide y and C27 from C1 using oligonucleotide z. Replacing the fragment a in C19 with CE3 generated C28. Finally, C29 was constructed from C28 by using oligonucleotide u. The VP1 effector construct (35S-Sh-Vp1) contains the coding sequence of Viviparous1 fused 5’ to the first intron of the maize sucrose synthase gene (ahf) and driven by the cauliflower mosaic virus 35S promoter (McCarty et al., 1991).

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members of the two different families expressed in aleurone cells. J. Biol. Chem. 263, 19953–19960.


Modular nature of abscisic acid (ABA) response complexes: composite promoter units that are necessary and sufficient for ABA induction of gene expression in barley.

Q Shen, P Zhang and T H Ho

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