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Functional definition of ABA-response complexes: the promoter units necessary and sufficient for ABA induction of gene expression in barley (*Hordeum vulgare* L.)

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Abstract

Abscisic acid (ABA)-response promoter complexes (ABRCs), consisting of an ACGT core-containing element (ACGT box) and a coupling element (CE), have been shown to be necessary and sufficient for ABA induction of gene expression in cereal plants. In this work, the component elements of two ABRCs are defined in terms of base sequence, orientation, and distance from each other. The ACGT element requires the sequence 5′-ACGTGGC-3′ and the elements CE1 and CE3 require the sequences CCACC and GCGTGTC, respectively. The ACGT element and CE3 are next to each other in the barley ABA-inducible gene *HVA1*, and lengthening the distance between them gradually decreases their activity in conferring ABA response. On the other hand, the ACGT element and CE1 are separated by about 20 bp in the promoter of another ABA-inducible gene, *HVA22*, and need to be separated by multiples of 10 bp in order to confer high ABA induction, suggesting that these two elements have to be located in the same side of the DNA double helix. Although the coupling between an ACGT box and a CE is sufficient for ABA induction, two copies of the ACGT element are equally active. However, two copies of CE3 appear to be less active. Specific interactions between ABRC and nuclear proteins have been detected. *In vitro* binding activities of nuclear proteins to an ABRC and to its mutant forms appear to be proportional to the biological activities of these sequences *in vivo*. Our data suggest that the specific response to ABA is determined by the presence of two ACGT boxes or an ACGT box plus a CE as well as by the flanking sequences of the ACGT boxes and the CEs.

Abbreviations: ABA, abscisic acid; ABRE, ABA-response element; ABRC, ABA-response complex; CE, coupling element

Introduction

Abscisic acid (ABA) mediates many developmental and physiological processes including seed development and stress responses. Intensive efforts in studying how plants respond to ABA have resulted in limited knowledge of its mode of action in regulating gene expression. Identification of *cis*- and *trans*-acting elements involved in the response of ABA-inducible genes remains an effective approach to decipher the steps of ABA signal transduction pathways. Deletion and linker-scan studies have been used to identify *cis*-acting elements designated ABREs (ABA-response elements) that are involved in ABA response in monocotyledoneous and dicotyledoneous species (Busk and Pagès, 1998 and references therein). ABREs contain an ACGT core, similar to the so-called G-boxes involved in responses to other environmental and physiological cues, such as light, anaerobiosis, auxin, jasmonic acid and salicylic acid (Foster *et al.*, 1994; Siberil *et al.*, 2001). Because of the similar structure

of ABREs and G-boxes, they are designated hereafter as ACGT boxes.

Studies with several promoters have led to the isolation of ACGT-box-binding proteins, all members of the bZIP-class DNA-binding proteins (Kim *et al.*, 1997; Hobo *et al.*, 1999b; Siberil *et al.*, 2001). The functional importance of ACGT boxes has been unequivocally demonstrated in ABA response. However, it remains a puzzle how similar *cis*-acting sequences specifically control response to diverse environmental and physiological signals. Two models have been proposed and studied. First, the bases flanking the ACGT core are involved in determining the signal response specificity of a promoter. This suggestion is based on the observation that binding patterns of nuclear extracts are determined by sequences flanking an ACGT core in gel mobility shift assays (Williams *et al.*, 1992). Furthermore, most bZIP proteins interact with ACGT-containing sequences with different affinities (Izawa *et al.*, 1993). Therefore, association of distinct bZIPs proteins with various types of ACGT boxes may regulate different signal transduction pathways. The second model suggests that it is the interaction of an ACGT box and a coupling element (CE) that determines specificity. In other words, ABA response relies on a response complex, namely ABRC (for ABA-response complex) (Shen and Ho, 1995; Shen *et al.*, 1996). In two ABA-inducible barley genes, *HVA1* (Hong *et al.*, 1992) and *HVA22* (Shen *et al.*, 1993), the ABRCs consist of an 8–10 bp element with an ACGT core (ACGT box) plus a CE (CE1 or CE3; Shen and Ho, 1995; Shen *et al.*, 1996). Specifically, the ABRC in *HVA22* (ABRC1) contains an ACGT box (A3, GCCACGTACA) and CE1 (TGCCACCGG), while that in *HVA1* (ABRC3) is composed of an ACGT box (A2, CCTACGTGGC) and CE3 (ACGCGTGTCCTC; Figure 1). CE3 in ABRC3 is located immediately upstream of the ACGT box, different from CE1 in the *HVA22* promoter in term of both its location and sequence. Exchange experiments demonstrated that an ACGT box could interact with either of these CEs to confer ABA response (Shen *et al.*, 1996). On the other hand, these CEs are not fully exchangeable, suggesting that the *HVA1* and *HVA22* genes contain distinct ABRCs (Shen *et al.*, 1996).

Similar configurations of an ACGT box plus a CE or two ACGT boxes are also found in other ABA-response cereal genes such as the wheat *Em* gene (Guiltinan *et al.*, 1990), and the rice genes *Rab16b* (Ono *et al.*, 1996), *Rab17* (Busk *et al.*, 1997), *Rab28* (Busk and Pagès, 1998) and *Osem* (Hattori *et al.*, 1995). These features in the promoters suggest that similar *trans*-acting factors may be mediating transcription of these genes. Based on studies with elements in the rice *Osem* promoter, it was proposed that ABRE3s and CE3 belongs to a single class of *cis*-acting elements (Hobo *et al.*, 1999a). Interestingly, although the CE does not have an ACGT core, both elements are similar in their sequences and can be recognized by the bZIP factor, TRAB1 (Hobo *et al.*, 1999b).

The two different models described above agree on the importance of ACGT boxes in mediating ABA response, yet differ concerning the elements that determine response specificity. Although more and more data suggest that ABA induction of gene expression relies on the presence of two *cis*-acting elements, it is also important to note that sequences flanking ACGT cores vary among ACGT boxes of ABA-responsive promoters. To further define the sequence of ABRC1 and ABRC3 essential for ABA response, we performed a series of point mutations in the ACGT boxes and CEs. We carried out gel retardation experiments with nuclear factors and *in vivo* function assays with different promoter constructs derived from an ABRC to understand the relative role of the *cis* elements. The effect of alternating the orientation of the ACGT box and CE, and varying the distance between ACGT boxes and CEs was also studied. These experiments have allowed us to clearly define the border of ACGT boxes and CEs in both ABRCs. In addition, our data indicate that ABRC1 and ABRC3 are not only different in term of their complex structures and CE sequences, but also in their orientation and distance requirement.

Materials and methods

Plant materials

Barley seeds (*Hordeum vulgare* L. cv. Himalaya) from the 1991 or 1998 harvests at Washington State University, Pullman, WA, were used in the experiments. For extraction of nuclear proteins, either aleurone layers or 2-day old seedlings were used. Aleurone layers were prepared from imbibed half seeds by removing the starchy endosperm and incubated as described before (Hong *et al.*, 1992). For treatment with ABA, aleurone layers or seedlings were incubated with 100 μM ABA for 14 h and continuous shaking.

Preparation of DNA constructs

The reporter constructs A22 (*ABRC1-GUS*) and A1 (*ABRC3-GUS*) correspond to the C17 and C1 constructs, respectively, described before (Shen *et al.*, 1996). Mutants of ABRC1 and ABRC3 were prepared by the method of oligonucleotide-directed mutagenesis as described elsewhere (Kunkel *et al.*, 1987) and detailed in Shen and Ho (1995) and Shen *et al.* (1996). The constructs G-box, A-box, C-box, 2xACGT and 2xCE3, mACGT, mCE3 and mACGTmCE3 were obtained by mutating the *ABRC3-GUS* construct.

Particle bombardment and transient expression assays

The detailed procedure of transient expression in barley aleurones by particle bombardment has been published previously (Shen *et al.*, 1993). Briefly, the mixture (in 1:1 molar ratio) of a test promoter-GUS reporter construct and a maize ubiquitin-luciferase internal control construct (3 μg each) was bombarded into barley embryo-less half seeds (four replicas per test construct). After incubation in the presence or absence of 20 μM ABA for 24 h, sets of four bombarded seeds were homogenized in 800 μl of the grinding buffer (Shen *et al.*, 1993). After centrifugation at 12 000 $\times$ *g* for 10 min at 4 °C, 100 μl of the supernatant was assayed for luciferase activity. For GUS assay, 50 μl of the supernatant was diluted with 200 μl of GUS assay buffer (Shen *et al.*, 1993) and incubated at 37 °C for 20 h. Then 50 μl of the reaction mixture was diluted with 2 ml of 0.2 M Na_2CO_3 and the resulting fluorescence was measured in a Sequoia-Turner 450 fluorometer adjusted to give a reading of 1000 units for 1 μM 5-methylumbelliferone. The normalized GUS activity represents the total number of fluorescent units in 20 h from an aliquot of extract that contained 2000 000 relative light units of luciferase activity.

Protein extracts and electrophoretic mobility shift assays

Nuclear proteins from aleurones or germinating embryos were obtained by first isolating nuclei by the method described (Luthe and Quatrano, 1980). The nuclear pellet was suspended in lysis buffer (20 mM Hepes-KOH pH 7.6, 100 mM KCl, 0.2 mM EDTA, 25% v/v glycerol, 1 mM dithiothreitol (DTT), 250 μM phenylmethylsulfonyl fluoride (PMSF) and 5 μg/ml leupeptin), and proteins were extracted by adding

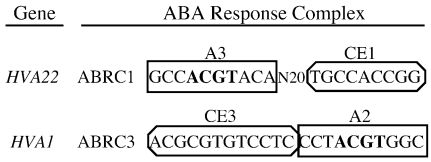

Figure 1. Basic features of the ABA response complexes (ABRCs) in the barley *HVA22* and *HVA1* genes. Each ABRC contains an ACGT box and a coupling element (CE). N20 indicates the 20 bp distance between A3 and CE1.

NH_4SO_4 to a final concentration of 0.5 M. After centrifugation at 40 000 $\times$ *g* for 30 min, nuclear proteins were precipitated with 350 mg/ml NH_4SO_4. The protein pellet was re-suspended and dialyzed in buffer D (20 mM Hepes-KOH pH 7.6, 50 mM KCl, 0.2 mM EDTA, 20% v/v glycerol, 1 mM DTT and 250 μM PMSF) and used for electrophoretic mobility shift assays. Partially purified DNA-binding proteins were also obtained from 2-day old embryos according to the method of Hollung *et al.* (1994), and enriched on a phosphocellulose column (Whatman P-11). Proteins were eluted with buffer D containing 800 mM KCl, dialyzed in buffer D and loaded onto a DEAE-Cellulose column (CELLEX-D, BioRad, Hercules, CA). The column was washed with buffer D containing 250 mM KCl, and the proteins were eluted with buffer D and 350 mM KCl and dialyzed again in buffer D before gel shift assays. 124 bp double-stranded oligonucleotide probes were obtained by digesting the constructs used in transient expression with *Not*I and *Xba*I, and by labeling them with ^{32}P-dCTP by the Klenow fill-in reaction. Binding reactions (20 μl) contained 1 ng of radiolabeled probe, 1 μg poly-dIdC, 10 mM Tris-HCl pH 7.6, 50 mM KCl, 0.5 mM EDTA, 10% v/v glycerol, 1 mM DTT, and 15 or 20 μg of protein extract, and were incubated at 4 °C for 30 min. Competition assays were carried out by adding 50- or 500-fold molar excess of unlabeled competitor. All reaction mixtures were resolved by electrophoresis on a 4% polyacrylamide gel in 0.5$\times$ TBE buffer.

Results

Comparison of promoter elements required for ABA induction of gene expression suggests the presence of unified ABA-response complexes in cereals. For instance, ABRC1 of the barley *HVA22* gene contains an ACGT box (A3, GCCACGTACA) and CE1 (TGCCACCG) located 20 bp apart (Figure 1). Interestingly, the wheat *Em* gene also contains two

similar ABA-response complexes. One includes the Em1b sequence (CACACGTGCC) and a CE1-like sequence (ACGCACCGC) located 16 bp downstream. Another ABRC may consist of Em1a, an ACGT box (GACACGTGGC) and Em2a (CGAGCAGGC), which is 16 bp downstream of Em1a and may serve as the CE. Similarly, the *HVA22* gene also contains a second ABRC, including the ACGT box (A2, CGCACGTGTC) and an Em2a-like sequence (CE2, CTAGCAGCC). These two elements are 28 bp apart and also have been demonstrated to confer a strong response to ABA (Shen and Ho, 1995). In the *HVA1* gene, ABRC3 is composed of an ACGT box (A2, CCTACGTGGC) and the adjacent CE3 (ACGCGTGTCCTC; Figure 1). An almost identical complex has been identified in the rice *Rab16b* gene, where the combination of Motifs I and II are essential for high ABA induction (Ono *et al.*, 1996). Motif I (AGTACGTGGC) is similar to the A2 of the barley ABRC3 and motif II (GCCGCGTGGC) is almost identical to barley CE3. At 104 bp upstream of this *Rab16b* ABRC, we noticed the presence of another ABRC, which consists of an ACGT box (AACACGTGCA) and a CE1-like sequence (CCTCACCGG). The aforementioned observations illustrate some examples of similar ABRC structures in cereal genes. However, we noticed the clear difference among CEs and sequences flanking the ACGT cores in all genes mentioned. Thus, it is necessary to investigate the sequence requirement in ACGT boxes and CEs crucial for ABA response.

Important sequence of the ACGT box in ABRC1 include the three bases upstream of the ACGT core

To determine the sequence of the ACGT box essential for ABA induction, we performed point mutations along the region of the A3 sequence in ABRC1. The data in Figure 2 indicate that the requirement for the ACGT core is absolutely stringent. Mutation of the adenine to any other three possible bases abolished the ABA response of the complex, with the induction dropping from 33-fold to only 3- or 4-fold (constructs 16–18). The effect of single point mutation on C, G or T base was just as severe (constructs 19–27).

The involvement of the sequence upstream of the ACGT core was also tested. The cytosine immediately upstream of the ACGT core was just as critical for ABA induction as any of the four core bases was. Mutation of this base abolished the ABA response of the complex (constructs 13–15). Mutation of the

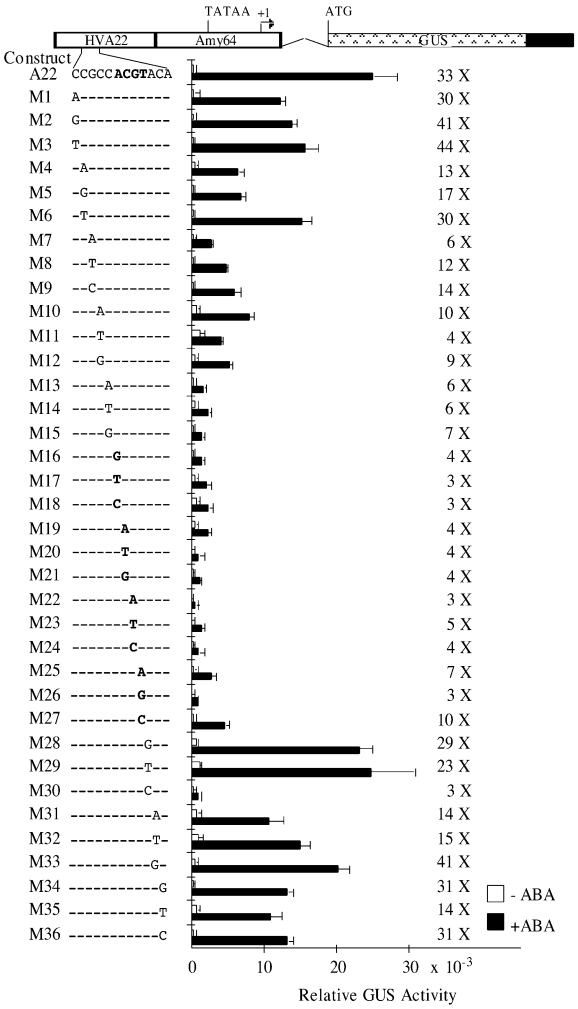

Figure 2. Mutation analyses of the ACGT element (A3) in the ABRC1 of the *HVA22* gene. A schematic diagram of the testing construct is shown at the top: the thin black angled line indicates the position of the intron1-exon2-intron2 fragment of *HVA22* inserted between the 5′-untranslated sequence (short solid box) and the GUS coding sequence (dotted box). The 3′ region (solid box) was from the *HVA22 Sph*I/*Sph*I genomic fragment that includes the polyadenylation sequence. The minimal promoter (open box) from the *Amy64* gene (extending from −60 to +57) provides the TATA box sequence. A 49 bp fragment of the *HVA22* promoter (from −104 to −56) was fused to the minimal promoter. The letter in the promoter sequence represents the bases that are mutated and the dash lines indicate the sequence similar to the wild type (A22). After bombardment, half seeds were incubated for 24 h with (solid bars) or without (open bars) 20 μM ABA. Bars indicate relative GUS activities ± standard error. X indicates fold of increase.

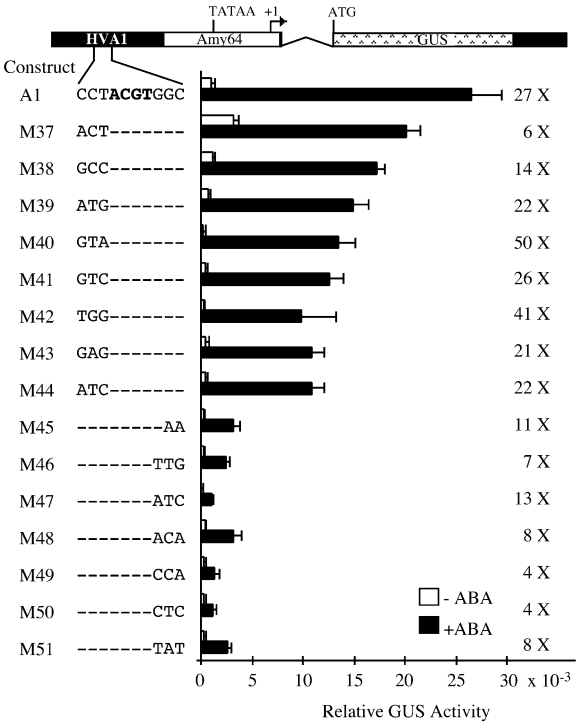

Figure 3. Mutation analyses of the ACGT element (A2) in the ABRC3 of the *HVA1* gene. The 68 bp fragment from the *HVA1* promoter (from −67 to −134, dark box) was fused to the progenitor construct MP64. All other labels are the same as in Figure 2. Bars indicate GUS activities ± standard error after 24 h of incubation of the bombarded seeds with (solid bars) or without (open bars) 20 μM ABA.

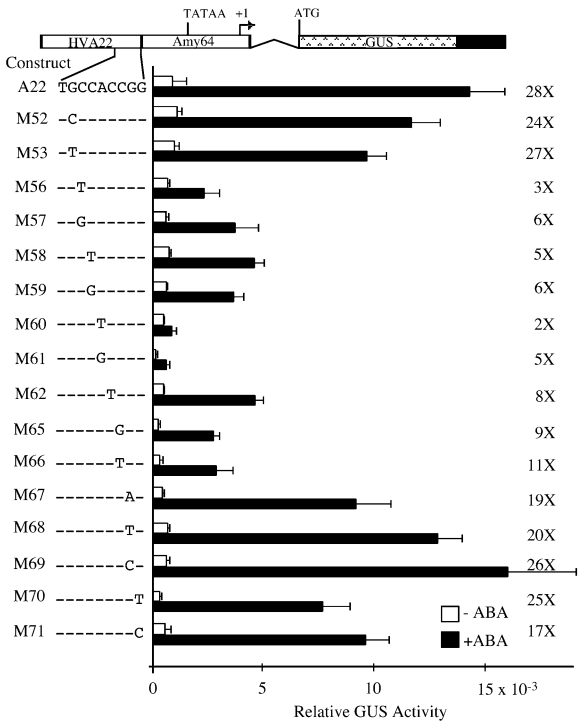

Figure 4. Mutation analyses of the CE1 in the ABRC1 of the *HVA22* gene. All labels are the same as in Figure 2. Bars indicate GUS activities ± standard error after 24 h of incubation of the bombarded seeds with (solid bars) or without (open bars) 20 μM ABA.

two bases further upstream also had significant effect (constructs 7–12). However, mutations of the first two bases had little effect (constructs 1–6). These results suggest that at least 3 bases upstream of the ACGT core are involved in the response of ABRC1 to ABA. In contrast, mutations of the sequence downstream of the ACGT core appeared to have much less effect. Eight out of nine point mutants conferred ABA response at a level comparable to that obtained with the wild-type ABRC1 (constructs 28–36). The exception was the adenine-to-cytosine mutation (construct 30) immediately flanking the ACGT core; only 3-fold induction was obtained compared to 33-fold of the wild-type complex. Taken together, the sequence requirement for the A3 element seems to be GCCACGT.

The essential sequence of the ACGT element in ABRC3 of HVA1 *is* ACGTGGC

Similar mutation experiments were carried out to define the border of the A2 element of ABRC3. Because our group and other investigators have demonstrated the importance of the ACGT core, no mutation was made in this core. Instead, the 5′ and 3′ sequences flanking the ACGT core were mutated. All eight tri-nucleotide mutants corresponding to the 5′-flanking sequence of A2 conferred significant ABA induction levels (constructs 37–44, Figure 3). Even the most detrimental mutation, CCT to ATC (construct 44) gave 22-fold ABA induction, with 50% of relative GUS activity as compared with that from the wild-type construct (construct A1). It is interesting to note that the mutant M42 has the same sequence than the construct M30 (Figure 2) but in inverted orientation. It is not clear why M30 presented such low ABA induction while in the ABRC1 context the inverted sequence (represented by M42) it showed relatively high GUS activity in response to ABA. In contrast, the 3′-ACGT core flanking sequence of A2 appeared

to be much more critical. Seven mutants (constructs 45–51), in which two or three nucleotides downstream from the ACGT core were replaced, showed much reduced ABA induction and absolute expression level. The ABA induction for these mutants ranged from 4- to 13-fold and expression levels were less than 15% of that obtained with the wild type. Hence, we concluded that the important region of A2 of *HVA1* is ACGTGGC.

The essential sequence of CE1 is CCACC

Our previous work demonstrated that the CEs in ABRC1 (CE1) and ABRC3 (CE3) are essential for ABA induction (Shen *et al.*, 1996). To further understand the structural nature of the two CEs, finer mutagenesis was carried out through the CE1 and CE3 regions. The most critical base in CE1 appeared to be the adenine in the middle of the element (Figure 4). When it was mutated to either T (construct 60) or G (construct 61), the ABA induction dropped to only 2- and 5- fold respectively, with their expression levels less than 5% of that obtained with the wild type. The 2 bp upstream from this central base also appeared to be necessary. Mutations of each of these two bases conferred less than 6-fold ABA induction (constructs 56–59). In contrast, when the nucleotide further upstream (G) was replaced with either C (construct 52) or T (construct 53), the ABA induction remained high (about 25-fold) and the expression level was more than 70% of that with the wild type. Mutation of the base immediately downstream from the core adenine base (C to T, construct 62) affected the ABA response (only 8-fold induction compared to 28-fold of the wild type). Similar reduction of both ABA induction and expression level was observed with the mutants of the next nucleotide (constructs 65 and 66). However, mutation of the two bases further downstream had much less effect (constructs 67–71). These data together suggest that the essential sequence in CE1 is CCACC.

The essential sequence of CE3 is GCGTGTC

Definition of the important sequence of CE3 was also performed to understand the differences between CE1 and CE3 and to identify similar CEs in other ABA inducible promoters. As shown in Figure 5, mutation of the first 2 bases of CE3 caused a reduction of the expression levels, but the induction by ABA remained significantly high (14- to 20-fold). Mutation of the first base (A to G, construct 73) reduced

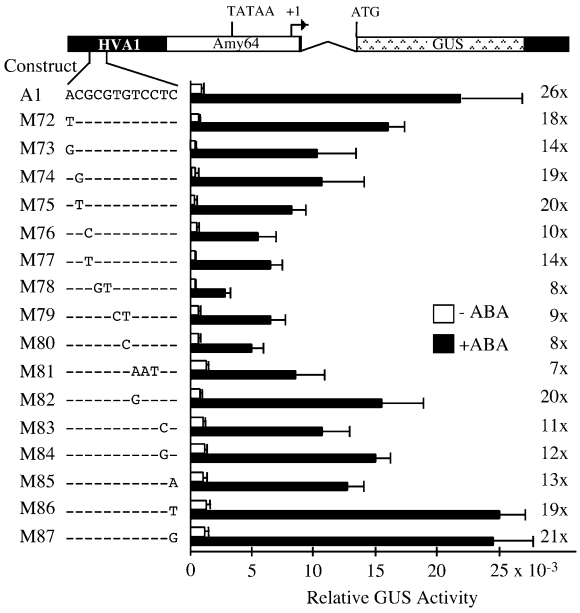

Figure 5. The definition of the CE3 in the ABRC3 of the *HVA1* gene. All other labels are the same as in Figure 3. Bars indicate GUS activities ± standard error after 24 h of incubation of the bombarded seeds with (solid bars) or without (open bars) 20 μm ABA.

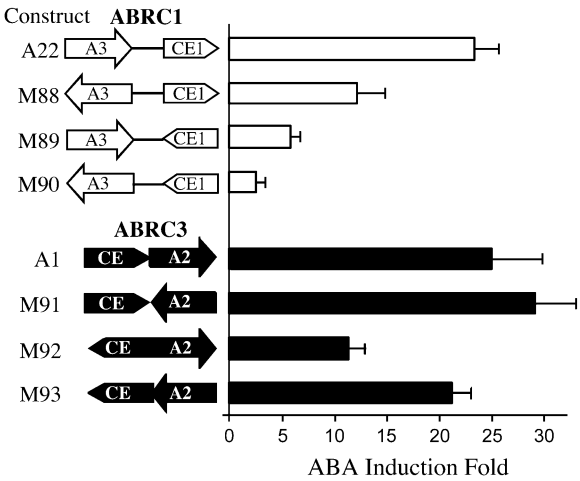

Figure 6. The relative orientation of the ACGT and CE elements is important for ABA induction. Constructs with the ACGT and coupling elements of ABRC1 (open bars) and ABRC3 (solid bars) in direct or reverse orientation were tested in transient expression assays. Bars indicate GUS activities ± standard error after 24 h of incubation of the bombarded seeds with 20 μM ABA.

the expression level to 50% of the wild type. A similar reduction resulted when the second base (C) was changed to G (construct 74) and T (construct 75) and when the third base was changed from G to C or T (constructs 76 and 77, respectively). The importance of bases 4–7 was demonstrated with double mutations (CG to GT, construct 78 and TG to CT, construct 79) and single mutations (G to C, construct 80). All of these three mutants conferred less than 10-fold induction and absolute expression levels lower than 30% of that with the wild type. A mutant that has bases 8–10 changed from TCC to AAT (construct 81) showed reduced ABA induction (from 26- to 7-fold) and expression level down to 40% of the wild type. The last two bases (TC) appeared be less critical (constructs 83–87). Taken together, we suggest that the critical sequence of CE3 is GCGTGTC.

Orientations required for elements in ABRC1 and ABRC3 are different

Even though an ACGT box can interact with a distal or proximal CE to confer similar level of ABA response (Shen *et al*., 1996), the interaction between these two *cis* elements is not similar in both ABRCs. The orientation of both elements is much critical in ABRC1, while the orientation of the ACGT box or CE3 in ABRC3 has less impact on the response to ABA. As shown in Figure 6, reversing the orientation of A3 (construct M88) or CE1 (construct M89) reduced the induction level to 52% and 26% of the wild type, respectively. When the orientation of both A3 and CE1 was reverted (construct M90), the induction dropped to 11%. These data suggest that both A3 and CE1 have to be properly oriented on the promoter to confer high ABA induction. In ABRC3, the elements A2 and CE3 were less sensitive to their orientation. Reversion of A2 (construct M91) resulted in high ABA induction. In contrast, when CE3 was reverted (construct M92), the induction level decreased to 44% of the wild type. When both elements were reverted (construct M93), the mutant complex still conferred high levels of induction.

Elements in ABRC1 are phase-sensitive while those in ABRC3 are distance-sensitive

ABRC1 and ABRC3 are not only different in term of their sensitivities to the orientation of their *cis*-acting elements, but also in the distance between the elements. Figure 7 shows the ABA induction level obtained with ABRC1 and ABRC3 when the distance

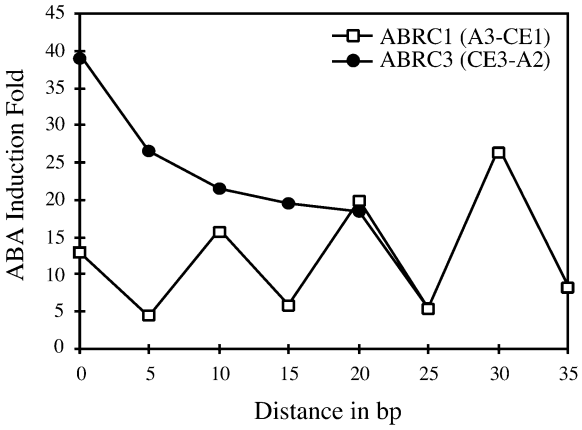

Figure 7. Effect of the distance between ACGT and CE elements on the ABA induction of ABRC1 and ABRC3. Constructs with increasing distance between the ACGT and coupling elements in ABRC1 (open symbols) and ABRC3 (closed symbols) were tested in transient expression assays. As shown in Figure 1, the original distance between A3 and CE1 is 20 bp and CE3 and A2 is 0 bp. Points represent the average fold increase of the relative GUS activity of samples from half seeds incubated in the presence of 20 μM ABA for 24 h and compared to that from samples incubated in the absence of ABA.

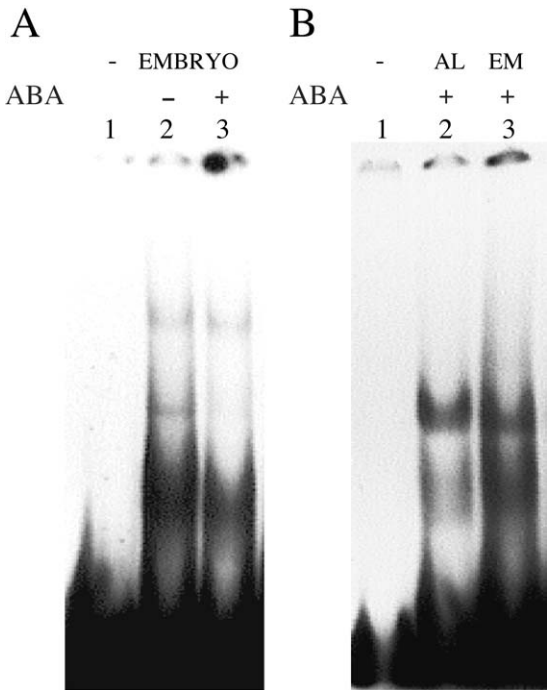

Figure 8. ABRC3 binding activity from barley embryos and aleurone layers. A. Electrophoretic mobility shift assay with a 124 bp fragment containing ABRC3 incubated alone (lane 1), or with 20 μg of nuclear proteins from non-ABA-treated (lane 2) or ABA-treated (lane 3) germinating embryos. B. Same assay with a different nuclear extraction: ABRC3 probe incubated alone (lane 1), or with extracts from ABA-treated aleurone layers (lane 2) or embryos (lane 3).

between the ACGT box and CE was altered. When A3 and CE1 were separated by multiples of 10 bp, the induction level was always higher than when they were separated by multiples of 5 bp. For instance, ABA induction was only 5-fold when A3 is 5 bp apart from CE1. When the distance increased to 10 bp, the ABA induction rose to 16-fold. When they were 30 bp apart, the induction level was as high as 26-fold, compared to 20-fold when 20 bp apart in the wild type. The induction level with a complex in which A3 and CE1 were separated by multiples of 10 bp was always higher than that when they were separated by multiples of 5 bp. Because most DNA in a physiological condition is in B-form conformation and each turn of B-from DNA consists of 10 bp, it is likely that the activity of ABRC1 relies on the in-phase interaction between factors binding to A3 and CE1.

In contrast, ABRC3, composed of A2 and CE3, does not appear to be phase-sensitive. The highest induction (about 40-fold) was obtained with the wild type complex, in which CE3 lies immediately upstream to A3. When a 5 bp sequence was inserted between the elements, the induction decreased to 26-fold. The induction level continued to decrease as the distance between A2 and CE3 was lengthened. When these two elements were 20 bp apart, the induction dropped to 19-fold. Further separation to 25 bp resulted in almost complete loss of induction (about 6-fold). Therefore, ABRC3 is distance-sensitive while ABRC1 is phase-sensitive.

Nuclear proteins recognize a specific ACGT box present in ABRC3

To further study the significance of the *cis*-elements in the ABRCs, *in vitro* assays were designed to analyze how these elements are recognized by nuclear factors. Nuclear proteins were extracted from germinating barley embryos and aleurone layers, because expression of *HVA1* and *HVA22* is induced in these tissues in response to ABA (Hong *et al*., 1992; Shen *et al*., 1993). Crude nuclear extracts obtained from both ABA-treated and control germinating embryos presented binding activity to the ABRC3 sequence (Figure 8A), indicating that ABA may not affect the presence or activity of the ABRC3 binding proteins. Comparable results were observed with extracts from aleurone layers (data not shown). In a separate extraction (Figure 8B), we showed that similar binding pattern was obtained with extracts from aleurones or germinating embryos. Further assays were performed using extracts from germinating embryos.

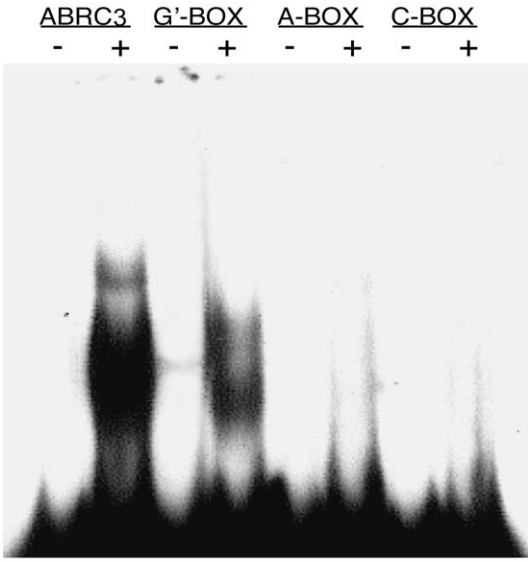

Figure 9. Specific binding activity to the ACGT box present in ABRC3. Electrophoretic mobility shift assay with a 124 bp fragment containing ABRC3 or fragments with mutations after the ACGT core were incubated with (+) or without (−) 15 μg partially purified nuclear proteins. The name of each oligonucleotide used as probe is indicated above the lanes and its partial sequence is shown below the figure.

To test whether the binding activity of the nuclear extracts was towards a specific ACGT box and not to any ACGT sequence, we performed binding assays with three DNA fragments that differ from the wild-type ABRC3 in only the 3 bases after the ACGT core (Figure 9). While the wild-type ABRC3 exhibited strong binding activity, a different G-box version (G′ box), with GAA right after the ACGT core, showed a moderately reduced binding activity. However, when the ACGT box was transformed to an A or C box (Foster *et al.*, 1994) by replacing the bases GGC by ATA or CCT, respectively, the binding activity was very weak.

Nuclear extracts recognize probes containing two cis *elements*

To elucidate the relative role of ACGT boxes and CEs in ABA response, mutated versions of ABRC3 and

other synthetic configurations were tested in gel shift assays. The *in vitro* binding activity of partially purified extracts was abolished when the ACGT box or both ACGT box and CE were mutated (Figure 10A). Only a weak interaction was observed when the CE was mutated, that is, the ACGT box remained intact. Other configurations tested included two copies of the ACGT box and two copies of the CE. In both cases the distance between the ACGT cores (or GCGT cores of CE3) were the same as in the wild type version. Two copies of ACGT box (2A) showed the same strong binding activity as the native ABRC3 sequence. However, two copies of the CE (2CE) displayed only a weak binding, similar to the mCE probe. Competition experiments with these new arrangements of *cis* elements were done to further prove the specific binding activity to the ABRC's elements (Figure 10B). As expected, ABRC3 and two copies of the ACGT box competed out ABRC3. mCE, which still has the ACGT box intact, also competed effectively. On the other hand, a fragment with only the CE or neither element could not compete out the wild-type ABRC3. According to its binding activity, two copies of CE3 only competed partially. In another competition assay, we found that the native ABRC3 could compete out both 2A and 2CE probes. 2A could compete out 2CE, but 2CE only competed partially both ABRC3 and 2A (data not shown). These data support the fact that the nuclear proteins bind 2CE with less affinity than to 2A.

In vitro *binding activity of ACGT- and CE3-containing promoters correlates with their activity* in vivo

All the DNA fragments used in *in vitro* binding assays were tested in a transient expression system. Promoter fragments containing the native ABRC3 or the sequences described in Figure 10C were fused to the GUS gene and delivered by particle bombardment into barley aleurone layers to test their response to ABA (Figure 10C). Two copies of ACGT box (2A) responded as well as the wild-type version of ABRC3 (25-fold induction), which correlates well with the strong *in vitro* binding activity. Similarly, the fragments G′ box and 2CE that presented a moderate binding activity, displayed a fair ABA response (around 10-fold induction). The rest of the fragments, A box, C box, mA, and mCE that showed weak *in vitro* binding activity, exhibited low induction levels (5-fold or lower). The response to ABA was abolished when both ACGT box and CE were mutated.

Discussion

In an effort to elucidate the molecular mechanisms mediating ABA response in cereals, we have extended our previous work to further define the promoter complexes required for ABA induction of gene expression. Specifically, we have defined the component elements of ABRCs in terms of their base sequences, orientation, and distance between them. We also demonstrated that the sequence required to confer ABA induction in the *HVA1* promoter, ABRC3, is recognized specifically by nuclear extracts from barley. The binding activity was specific for the class of ACGT box present in ABRC3 and recognized the wild type version of the ABRC, two ACGT boxes, and with less affinity two copies of the CE. In addition, we showed that this *in vitro* activity correlates positively with the *in vivo* activity of the promoter constructs, which also suggests that the binding activity observed is probably needed for the ABA induction of gene expression *in vivo*.

The data presented here serve to explain the specific response mediated by ACGT boxes. CEs and sequences flanking ACGT cores have been suggested to determine the response to signals such ABA, blue and white lights, and coumaric acid (Block *et al.*, 1990; Donald and Cashmore, 1990; Loake *et al.*, 1992; Williams *et al.*, 1992; Foster *et al.*, 1994; Shen and Ho, 1995; Shen *et al.*, 1996). Functional definition of the ACGT boxes described in this work provides additional support for the hypothesis that sequences flanking the ACGT core contribute to specificity for the response to ABA. Our study indicates that the important conserved sequence is ACGTGGC, either in a direct (as in ABRC3) or an inverted orientation (as in ABRC1). Nine of 27 possible mutations of the GGC sequence downstream from the ACGT core have been analyzed in this study. All these nine mutations in the A3 (Figure 2), in addition to the mutations in A2 to TTG, ATC, ACA, CCA, CTC, TAT shown in Figure 3 and ATA, CCT and GAA shown in Figure 10C, drastically reduced the ABA response of ABRC3. Similarly, all nine possible single nucleotide mutations of the corresponding nucleotides in ABRC1 had a detrimental effect (Figure 2, constructs 7–15). This ACGT box sequence agrees with the essential sequence for the ACGT box of the *Osem* promoter as reported recently (Hattori *et al.*, 2002). The requirement for such ACGT box was determined as ACGTGGC or ACGTGTC, which also coincides with the consensus from sequences in ABA-responsive promoters. Not-

ably, the construct M10 that has GCCACGT changed to GACACGT also presented a significant ABA response, similar to mutants M4 and M5 that have mutations in the fourth base from the ACGT core (Figure 2). Together, these results suggest an asymmetric sequence requirement for the ACGT boxes of ABRCs, which interact with bZIP proteins (Hattori *et al.*, 1995). Since bZIP proteins can form both homodimers and heterodimers to bind to a target ACGT box (Schindler *et al.*, 1992; Nantel and Quatrano, 1996), it is plausible that a heterodimeric complex instead of homodimeric complexes bind to specific ACGT boxes to confer ABA response. It should be emphasized, however, that not every ACGT box is involved in ABA response, as clearly shown in Figures 3 and 10C. This is a point to consider as some current bioinformatic work simply relies on conserved core sequence information.

We proposed the presence of a CE to specify ABA responsiveness in combination with an ACGT box (Shen and Ho, 1995; Shen *et al.*, 1996). Now it is accepted that a single copy of an ACGT box cannot confer ABA responsiveness. ABA-responsive promoters present either perfect palindromic ACGT boxes (CCACGTGG), imperfect or hybrid ACGT boxes (i.e. ACGTGTC) or non-ACGT *cis* elements such as CE3 (GCGTGTC). The sequence of CE3 differs from that of an ACGT box only in the base at position –1 (G instead of A). It has been shown that similar CE can function as another ACGT box in the rice *Osem* promoter (Hobo *et al.*, 1999a). It is also interesting to note that similarly to an ACGT box, CE3 can function as GCGTGTC (wild type) or GCGTGGC (construct 82, Figure 5) to confer ABA response.

The presence of an ACGT box and a CE3-like sequence has been reported in several promoters. The CE3-like sequences in the *Rab16* (GCCGCGTGGC; Ono *et al.*, 1996) and *Osem* (ACGGCGTGTC; Hobo *et al.*, 1999a) promoters were also shown to be important to confer ABA-inducible expression. The location of these elements, however, varies in these promoters. In the *HVA1* promoter, CE3 is located right upstream of the accompanying ACGT box. In the *Rab16b* and *Osem* promoters they are located 8 and 21 bp downstream of the ACGT box, respectively. Transient expression studies presented here suggest that a promoter containing either one ACGT box plus a CE or two ACGT boxes has a high response to ABA, similar to what was suggested for the *Rab16b* and *Osem* genes. However, in our case two copies of CE3 did not respond as well as two copies of the ACGT box when tested in transient expression assays, as it was reported for the *Rab16b* and *Osem* promoter elements (Ono *et al.*, 1996; Hobo *et al.*, 1999a). One explanation is that the distance requirement for two coupling elements may differ from that for the wild-type configuration in order to be recognized by the same binding proteins. Hobo *et al.* (1999b) demonstrated that the bZIP factor TRAB1 recognizes both the ACGT box and the CE3 of the *Osem* promoter. Our *in vitro* assays, however, indicate that the specific nuclear activity had less affinity to CE3 than to the ACGT box. Similarly, our *in vitro* assays showed that mutation of either the ACGT box or CE3 altered the binding of nuclear proteins (Figure 10A). In addition, the barley bZIP HvABI5 has been show to recognize the ACGT box and CE3 of ABRC3 and also presents more affinity towards the ACGT box than to CE3 (Casaretto and Ho, 2003). Nevertheless, HvABI5 requires the presence of the ACGT box and CE3 to fully trans-activate ABRC3 *in vivo* (Casaretto and Ho, unpublished results).

The importance of the distal CE1 in mediating the ABA response of ABRC1 is further demonstrated here. A single point mutation (A to T) completely abolished the response to ABA (construct 60, Figure 4). Data from other fifteen mutants have allowed us to narrow down the CE1 element from the previously published 9 bp fragment, TGCCACCGG, to be a 5 bp fragment, CCACC. Interestingly, similar sequences containing a CACC core have been found in other ABA-responsive genes such as *Rab17*, *Rab16A*, *Rab16B*, *Rab16C*, *Rab16D*, *cDeT27-45*, *Em*, *LE25*, *Atrab18* and *Adh1* (Niu *et al.*, 2002; Shen and Ho, 1995; and references therein). The role of CE1- like sequences has been studied in some of these promoters. A CCACCG sequence is located right downstream of a CE3 element in the *Rab16B* promoter. Mutation of CE3 but not of the CE1-like sequence has effect on the response of the promoter to ABA (Ono *et al.*, 1996). Similarly, in *HVA1* a CACCG sequence is located downstream of A2 and mutation of this sequence does not affect the ABA induction of the promoter (Straub *et al.*, 1994). In the *Rab17* promoter, however, the DRE2 element (CCACCGAC) containing a CE1-like sequence is required for the response of the promoter to ABA (Busk *et al.*, 1997). In this case, the recognition may be carried out by the DREB factors (Narusaka *et al.*, 2003). A maize homologue of the *Arabidopsis ABI4* gene (*ZmABI4*), encoding an AP2 domain transcription factor, has been found to bind CE1-like sequences (Niu *et al.*, 2002). Even though this factor was shown to bind several CE1-

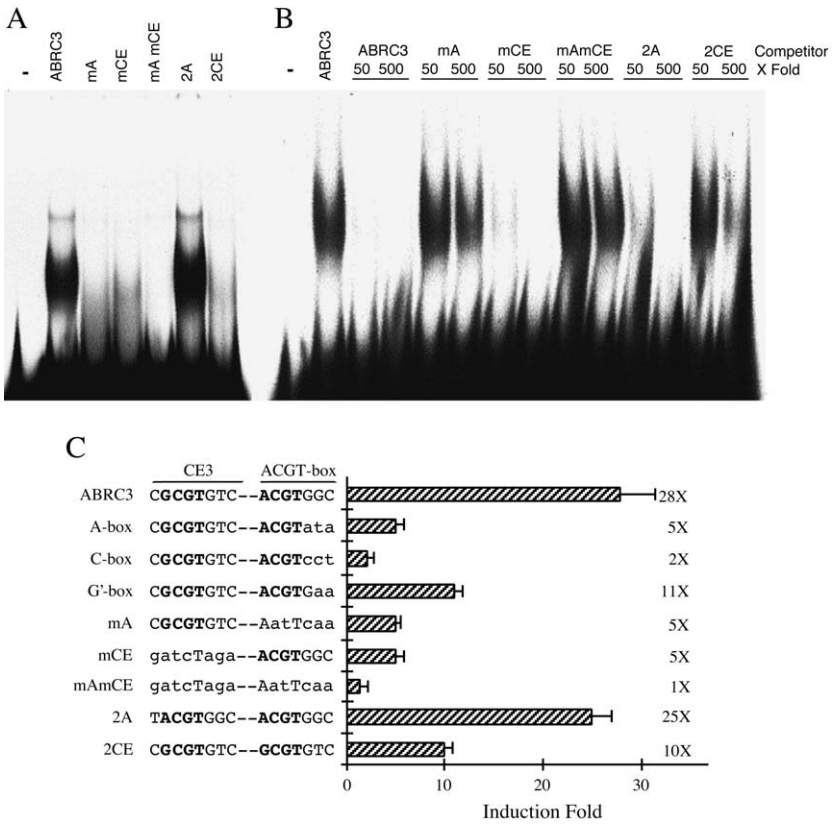

Figure 10. Two *cis* elements in an ABRC are required for *in vitro* and *in vivo* activity. All binding reactions contain 15 μg of partially purified nuclear proteins. A. Electrophoretic mobility shift assays with 124 bp fragments containing ABRC3, mutated versions of it, two copies of the ACGT box (2A) or two copies of CE3 (2CE). B. Competition assays with one or two copies of the ACGT box or CE. A probe containing ABRC3 was used in binding assays and competed out with itself, mutated versions of ABRC3, two copies of the ACGT box (2A) or CE3 (2CE). 50- or 500- fold excess of competitor was used. C. Comparison of ABA responsiveness of different configurations of the ABRC3 promoter region in transient expression assays. Partial sequence of DNA fragments used in binding and transient expression assays corresponding to the CE and ACGT box regions are described on the left. The fragments were fused to the *GUS* reporter gene and used for particle bombardment on barley aleurone layers. Bars represent the average fold increase $\pm$ standard error of relative GUS activity of samples from half seeds incubated in the presence of 20 μM ABA for 24 h and compared to that from samples incubated in the absence of ABA.

like sequences with the CACCG consensus core, it is important to mention that ZmABI4 recognizes those elements with different affinities and that such interactions has been proven only *in vitro* (Niu *et al*., 2002). It would be important to determine whether ABI4 homologues play a role in mediating ABA response of the *HVA22* promoter as well as other CE1-containing promoters *in vivo*. Probably an AP2 domain transcription factor other than ABI4 may recognize CE1 of *HVA22*. This possibility is based on the observation that although overlapping, the required sequence is CCACC and not CACCG. AP2 domain transcription factors are also known to bind different consensus sequences such as DRE elements (CCGAC; Narusaka *et al*., 2003). In this regard, it is worthwhile to point out that in CE1, mutation of any cytosine base in the CCACC core (constructs M56-59 and M62-66) rendered a partially functional element with a low ABA induction. Only the central adenine seems to be crucial for a functional CE. This may be due to low affinity recognition of a target site containing three Cs flanking the purine base by an AP domain transcription factor.

An ABA-responsive promoter, then, may present a combination of two *cis* elements, with at least one target for bZIP factors. For instance, many drought inducible genes contain a drought response element (DRE) and an ABA response element (ACGT box). Recently, it has been proposed that DRE motifs can function as coupling elements of an ACGT box (Narusaka *et al.*, 2003). DRE-binding proteins (also members of the AP2 class of transcription factors) and bZIP factors (homologous to TRAB1 and HvABI5)

have been shown to act synergistically in the transactivation of the ABA inducible gene *rd29A* (Narusaka *et al.*, 2003), suggesting that DREs and ACGT boxes depend on each other for the ABA response.

The orientation of the ACGT box and CE1 in ABRC1 is important for a high level of ABA response. Surprisingly, reverting the orientation of the ACGT box (construct M88, Figure 6) A2, caused a 50% reduction of the ABA induction. It should be noted that an A2-CE1 construct has been shown to confer a high ABA response (Shen *et al*., 1996). Reverting the orientation of CE1 was more critical (Figure 6). It is plausible that the ACGT box-binding bZIP protein complex interacts directly with the factor binding the CE1 element. In this regard, it is intriguing to note that the ABA induction is always higher when the ACGT box and CE1 are separated by 10, 20 or 30 bp than when they are separated by 5, 15 or 25 bp (Figure 7). It appears that the proteins on the ACGT box (a bZIP) and CE1 (possible an AP2 class factor) have to be located in the same side of the DNA helix in order to interact with each other. We cannot rule out the possibility, however, that A3 and CE1 need to be in opposite phase. If the three bases downstream the ACGT core (ACA) and the two before the CCACC core (TG), which do not seem to be important for ABA response, are counted in the distance between the ACGT and CCACC cores, then the distance between them would be 25 bp instead of 20 bp. In such case, distances of 5, 15, 25 and 35 bp results in high ABA induction. Nonetheless, the number of bases flanking the elements' cores to confer high ABA response is unknown. Changing the orientation of the ABRC elements or positioning the binding protein complexes in different phase would prevent the interaction of the two factors, hence reducing ABA induction. In contrast to ABRC1, the orientation requirement of the ABRC3 components appeared more flexible. Changing the orientation of the ACGT box in ABRC3 (A2) or both A2 and CE3 had no effect on ABA response and reversing CE3 exhibited a reduced induction level (44% of the wild type; Figure 6). Considering that the binding of nuclear proteins to ABRC3 relies on intact A2 and CE3 elements (Figure 10), it is possible that the interaction of A2- and CE3-binding proteins is mediated by other protein(s). In accordance with this hypothesis, it has been shown that the ABA response of ABRC3 is dependent on the presence of the barley VP1 protein (Casaretto and Ho, 2003). Possibly, the size of the protein (or protein complexes) is large enough so that it is able to mediate the interaction of A2- and CE3-binding factors like the bZIP HvABI5 (Casaretto and Ho, 2003) even when the A2 element is inverted. This is supported by the observation that ABRC3 is phase-insensitive but distance-sensitive. The induction level was high at short distances (Figure 7), but a drastic reduction was observed when the distance was increased to 25 bp, implying a threshold for the interaction of A2- and CE3-binding factors. We have also found that A2 and CE3 function not only as ABA-response elements, but also as elements required for the regulation by the co-activator VP1 (unpublished data), which is also known to interact with TRAB1 (homologue of HvABI5; Hobo *et al.*, 1999b). All these data suggest that VP1 interacts with the ABRC-binding proteins.

One important consideration when working with DNA-binding proteins is that *in vitro* binding assays often do not reflect the actual interaction that may be taking place *in vivo*. Hence, it becomes important to include an *in vivo* assay to explain the activity of the *cis* elements and *trans*-acting factors. Since ACGT boxes can be recognized readily by a variety of bZIP transcription factors, the first concern was to determine the specificity of the nuclear proteins to the type of ACGT box in the ABRC. Both *in vitro* (Figure 9) and *in vivo* (Figure 10A) experiments clearly indicated that more activity is achieved with the wild-type version of the ABRC3 and less than half when a different G-box is utilized. Only minor interactions can be detected when bases outside the ACGT core are changed (i.e. to an A box or C box; Figure 9). These results clearly demonstrate that the *in vivo* activities of the promoter constructs tested in the transient expression system correlate positively with those observed in the binding assays.

Although we have candidates for the formation of an ABRC-protein complex, it is not totally clear how ABA affects the formation of the complex, nor the modification of its components. Phosphorylation can modulate either positively or negatively the DNA-binding activity of transcription factors, or can affect the interaction of transcription factor transactivation domains with the transcriptional machinery (Schwechheimer and Bevan, 1998). Phosphorylation of the *Arabidopsis* G-box binding factor GBF1 by a casein kinase II stimulates its binding activity (Klimczak *et al*., 1992). Similarly to what was observed with aleurone and embryo extracts (Figure 8), both non-ABA-treated and ABA-treated tissues have been shown to contain nuclear factors capable of binding ACGT boxes (Guiltinan *et al.*, 1990; Hollung *et al.*,

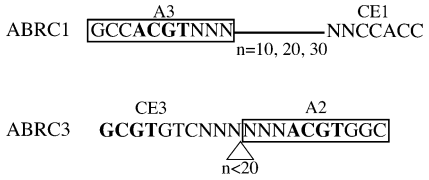

Figure 11. Summary of the features of the ABA response complexes (ABRCs) in the *HVA22* and *HVA1* genes. The required sequence for each *cis* element is described. N denotes any nucleotide and *n* the distance in bp between the elements.

1997). This suggests that a modification of the promoter complex, such as phosphorylation, would be important in ABA-induced transcription. In addition, the expression of certain transcription factors may be regulated by ABA as well. Expression of *EmBP1* and *VP1* are ABA-independent, while bZIP factors such as *LIP19*, *OSBZ8*, *TRAB1*, *MLIP15* and *HvABI5* are upregulated by ABA (Aguan *et al.*, 1993; Kusano *et al.*, 1995; Nakagawa *et al.*, 1996; Hobo *et al.*, 1999b; Casaretto and Ho, 2003). For example, expression of *TRAB1*, which recognizes an ACGT box and CE3 in the *Osem* promoter, is slightly induced by ABA (Hobo *et al.*, 1999b) and is also phosphorylated by an ABA-dependent signal (Kagaya *et al.*, 2002).

Functional analyses of the components of the ABRCs presented in this work indicate that both the sequences flanking ACGT cores and the CEs are important factors in determining the specificity and response level of promoter elements to ABA. As described in Figure 11, the important sequence in both ACGT boxes is ACGTGGC. In ABRC1, the ACGT box and CE1 must be in a particular orientation and distance from each other to confer high ABA induction. In ABRC3, however, the ACGT box can function in either orientation, and the response to ABA diminishes as the distance between the elements increases.

The information about the promoter elements described in this study could be helpful for the expression of genes that confer plant stress tolerance. Plant tolerance to environmental stresses has been achieved by over-expressing a bacterial gene encoding mannitol-1-phosphate dehydrogenase (Tarczynski *et al.*, 1993), the barley *HVA1* gene (Xu *et al.*, 1996), a yeast gene encoding a trehalose-6-phosphate synthase (Yeo *et al.*, 2000), and a mothbean pyrroline-5-carboxylate synthetase gene (Kishor *et al.*, 1995). However, expression of these genes was driven by constitutively active CaMV 35S or actin promoters. Because environmental stresses including salinity, cold and drought result in increased ABA levels in the plant cell, substitution of constitutive promoter with one ABRC-derived synthetic promoter would result in a gene which expression is under the control of a stress-responsive molecular switch. These synthetic promoters operate in vegetative tissues, as demonstrated in transiently transformed barley leaves (Shen *et al.*, 1996) and stable transformed rice plants (Su *et al.*, 1998), which makes more feasible their application in biotechnology.

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