Plant Molecular Biology **47:** 437–448, 2001.
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Dissection of abscisic acid signal transduction pathways in barley aleurone layers

Qingxi Shen[1], Aurelio Gomez-Cadenas[1,4], Pengnian Zhang[1], Mary Kay Walker-Simmons[2], Jen Sheen[3] and Tuan-Hua David Ho[1,*]

[1]*Plant Biology Program, Department of Biology, Division of Biology and Biomedical Sciences, Washington University, St. Louis, MO 63130, USA (*author for correspondence; e-mail ho@biology.wustl.edu;* [2]*USDA-Agriculture Research Service, Washington State University, Pullman, WA 99164-6420, USA;*
[3]*Department of Molecular Biology, Massachusetts General Hospital and Department of Genetics, Harvard Medical School, Boston, MA 02114, USA;*
[4]*Current address: Department of Experimental Sciences, Universitat Jaume I, 12071 Castellón, Spain*

Received 21 September 2000; accepted in revised form 31 May 2001

Key words: ABA signal transduction, ABI1, α-amylase, barley aleurone layer, PKABA1, VP1

Abstract

Abscisic acid (ABA) induces genes that are highly expressed during late embryogenesis, but suppresses gibberellin (GA)-responsive genes essential for seed germination and seedling growth. Promoter elements necessary and sufficient for ABA up- and down-regulation of gene expression have been previously defined in barley aleurone layers. We have studied the effect of a protein phosphatase 2C, ABI1, an ABA-inducible protein kinase, PKABA1, and a transcription factor, VP1, on ABA action in a barley aleurone transient expression system. The observations have allowed us to dissect ABA signal transduction pathways leading to either induction or suppression of gene expression. The ABA induction of embryogenesis genes is highly inhibited in the presence of a mutated protein phosphatase 2C, encoded by the *abi1-1* dominant mutant gene that is known to block ABA responses in *Arabidopsis*. However, the *abi1-1* gene product has no effect on the ABA suppression of a GA-responsive α-amylase gene. On the other hand, PKABA1 suppresses the expression of α-amylase genes, but has little effect on ABA up-regulated genes. Therefore, it appears that ABA induction and suppression follow two separate signal transduction pathways with the former inhibited by ABI1 and the latter modulated by PKABA1. The presence of VP1 enhances the ABA induction of late embryogenesis genes, but also suppresses germination specific genes. A schematic model based on these observations is presented to explain the effect of these regulatory proteins on ABA-mediated gene expression.

Abbreviations: ABA, abscisic acid; *ABI1*, ABA-insensitive 1 gene; ABI1, protein encoded by *ABI1*; *abi1-1*, dominant mutant gene of *ABI1*; ABRC, ABA response complex; ACGT-box, ACGT-core-containing box; CDPK, calcium-dependent protein kinase; CE, coupling element; GA, gibberellins; GA_3, gibberellic acid; GARC, GA response complex; GUS, β-glucuronidase; LEA, late embryogenesis-abundant; PKABA1, ABA-inducible protein kinase; *VP1*, *viviparous 1* gene; VP1, protein encoded by *VP1*

Introduction

The phytohormone abscisic acid (ABA) regulates several aspects of plant development such as seed formation, dormancy and germination, as well as plant responses to environmental stress conditions such as drought, cold and salinity (Zeevaart and Creelmann, 1988). At least part of the ABA action is mediated via alterations of gene expression (Skriver and Mundy, 1990; Chandler and Robertson, 1994; Busk and Pagès, 1998). Many genes which are highly expressed in

seeds during late embryogenesis and/or in stressed tissues are also induced by ABA (Vicient *et al.*, 2000). On the other hand, genes expressed in germinating seeds and seedlings, such as those encoding enzymes for starch and storage protein degradation, are induced by gibberellins (GA), but suppressed by ABA (Jacobsen *et al.*, 1995).

Analyses of ABA-induced gene promoters have revealed that a 10 bp element containing an ACGT core (ACGT-box, also named G-box or ABRE in published reports) is essential for ABA response (Marcotte *et al.*, 1989; Michel *et al.*, 1993; Shen *et al.*, 1993). In a series of mutational analyses of two ABA-responsive barley genes, *HVA1* and *HVA22*, it was shown that in addition to the ACGT-box, a coupling element (CE) is also necessary for ABA response (Shen and Ho, 1995; Shen *et al.*, 1996). The combination of the ACGT-box and the CE forms an ABA response complex (ABRC) which has been shown to be the smallest ABA-responsive promoter unit (Shen *et al.*, 1996). One copy of ABRC is sufficient to confer more than 30-fold ABA induction of the *GUS* reporter gene when linked to a minimal promoter. Mutations in either the ACGT-box or the CE practically abolish the ABA response (Shen and Ho, 1995).

The ABA suppression of genes encoding enzymes important for seed germination and seedling growth, such as α-amylases and proteases, has been mainly studied in cereal aleurone layers. Apparent differences between ABA-mediated induction and suppression of genes have been observed. First, no ACGT-box seems to be involved in ABA suppression (Rogers and Rogers, 1992). Second, while ABA suppresses the GA induction of α-amylase and proteases, GA usually has no effect on the ABA induced genes.

The signal transduction pathway leading to ABA regulated gene expression has not been fully explored. The initial perception of ABA has been suggested to take place on the outside surface of plasma membranes (Gilroy and Jones, 1994), although an internal receptor has not been ruled out (Schwartz *et al.*, 1994). Changes in cytosolic free Ca^{2+} levels and pH seem to act as intermediaries of the ABA signal transduction in different plant tissues (Wu *et al.*, 1997; Leung and Giraudat, 1998). Reductions of Ca^{2+} levels and increases in pH in response to ABA treatment have been reported in barley aleurone protoplasts (Wang *et al.*, 1991; van der Veen *et al.*, 1992). Furthermore, Ca^{2+} has been proposed to be involved specifically in the ABA down-regulation of GA-induced events while the ABA up-regulation of gene expression could be Ca^{2+}-independent (Gilroy, 1996).

Genetic analyses have revealed the role of other regulatory molecules in mediating ABA-regulated gene expression. Mutations in the *Viviparous 1* (*VP1*) gene in maize leads to precocious germination. Although the level of ABA is unaffected in *vp1* mutants, their sensitivity to ABA is much reduced (Robichaud *et al.*, 1980). McCarty *et al.* (1991) have shown that VP1 modifies both ABA up- and down-regulated gene expression. The VP1 protein contains a transactivation domain for gene regulation and it is likely to interact with other factors important for the ABA induction of *Em* gene (Schultz *et al.*, 1998). The *Arabidopsis* ABA-insensitive 1 (*ABI1*) gene codes for a protein phosphatase 2C (PP2C) involved in ABA signal transduction (Leung *et al.*, 1994; Meyer *et al.*, 1994). The role of PP2C mediating ABA regulation of gene expression has also been shown in maize leaf protoplasts (Sheen, 1998). Recently, it has been suggested that ABI1 is a negative regulator of ABA signaling (Gosti *et al.*, 1999). Furthermore, protein farnesylation, a post-translational modification process, is also important in mediating ABA sensitivity (Cutler *et al.*, 1996). A protein farnesyl transferase, encoded by the *ERA1* gene, acts downstream of *ABI1* in *Arabidopsis* guard cells (Pei *et al.*, 1998). The *SPY* gene, probably involved in post-translational modifications of proteins, has also been shown to affect ABA- and GA-regulated gene expression (Robertson *et al.*, 1998). Protein phosphorylation is also involved in ABA signaling (see Leung and Giraudat, 1998; Li *et al.*, 2000). In maize leaf protoplasts, two Ca^{2+}-dependent protein kinases (CDPK) are able to transactivate the expression of an ABA-inducible gene in the absence of the hormone (Sheen, 1996). In aleurone layers, an ABA-inducible protein kinase, PKABA1 (Anderberg and Walker-Simmons, 1992) can specifically suppress the GA induction of hydrolytic enzymes (Gomez-Cadenas *et al.*, 1999). Finally, phospholipase D has been implied as an intermediate in ABA signal transduction in barley aleurone layers (Ritchie and Gilroy, 1998).

Several components of the ABA signal transduction have been identified, but the interactions among them in mediating ABA-regulated gene expression are not fully understood. In this work, we have co-expressed ABA-regulated reporter constructs with effector constructs encoding ABI1, VP1, and PKABA1 in barley aleurone layers to better define the signal transduction pathways mediating ABA induction and suppression of gene expression. Our results suggest

that ABA-regulated gene expression branches into two separate signal transduction pathways with the ABA induction pathway sensitive to ABI1 protein phosphatase 2C and its dominant negative mutant, and the suppression pathway modulated by PKABA1.

Materials and methods

Preparation of DNA constructs

The reporter constructs were prepared as follows. First, ABRC1-GUS was constructed by linking the 49 bp ABRC1 to the progenitor, MP64, which was obtained by fusing the truncated (−60) promoter of *Amy64* gene (Khursheed and Rogers, 1988), and its 5′-untranslated region (downstream to +57 relative to the transcription start site), to a construct containing *HVA22* intron1-exon2-intron2, the *Escherichia coli* β-glucuronidase (GUS) coding region, and *HVA22* 3′ region (Shen and Ho, 1995). Next, ABRC3-GUS was made by ligating a 68 bp fragment containing ABRC3 into the *Sma*I-digested MP64 progenitor (Shen *et al.*, 1996). GARC(l)-GUS included the promoter (up to −331), the entire 5′-untranslated sequence and the first intron of a low-pI α-amylase gene, *Amy32b*, fused to the GUS coding sequence and the 3′-untranslated region of the same α-amylase gene (Lanahan *et al.*, 1992). GARC(h)-GUS was constructed by linking the promoter (to −400), the entire 5′-untranslated sequence, and the first intron of the high-pI α-amylase gene, *Amy 64*, to the GUS coding sequence and the 3′-untranslated region of the *Amy 4-6* gene (obtained from J.C. Rogers, Washington State University, Pullman, WA, USA).

The effector constructs have been described elsewhere. Briefly, 35S-ABI1, 35S-abi1-1, 35S-CDPK1, 35S-CDPK1a, and 35S-null-CDPK1 were made by linking the cauliflower mosaic virus 35S promoter, the 5′-untranslated region of the maize pyruvate orthophosphate dikinase gene, *C4ppdkZm1*, and the 3′-untranslated sequence of the *Agrobacterium* nopaline synthase gene (*Nos*) (Sheen, 1993) to the coding region of several *Arabidopsis* proteins: *abi1-1* dominant negative mutant gene, wild type *ABI1* gene (Leung *et al.*, 1994; Armstrong *et al.*, 1995), *CDPK1*, *CDPK1a*, or *null-CDPK1* (Sheen, 1996, 1998). 35S-ZmPP1 was made by replacing the *abi1-1* coding sequence of the 35S-abi1-1 construct with that of the maize type 1 protein phosphatase gene, *ZmPP1* (Smith and Walker, 1991). Finally, the preparation of the 35S-VP1 construct has been described by McCarty *et al.* (1991) and 35S-PKABA1 and 35S-CDPK1ci are detailed in Gomez-Cadenas *et al.* (1999) and Harper *et al.* (1994).

Particle bombardment and transient expression assays

The detailed procedure of transient expression studies with the barley (*Hordeum vulgare* L.) aleurone system and the particle bombardment technique have been published before (Lanahan *et al.*, 1992; Shen *et al.*, 1993). Briefly, the mixture (in 1:1 molar ratio) of a reporter construct and a maize ubiquitin/luciferase internal control construct (Ub-LUC) (Bruce *et al.*, 1989) was bombarded into barley embryoless half seeds (four replicates per test construct). Effector constructs were included as indicated in individual figures. After incubation in the presence or absence of 20 μM ABA or 1 μM gibberellic acid (GA_3) for 24 h, the bombarded seeds in sets of four were homogenized in 800 μl of the grinding buffer (Shen *et al.*, 1993). After centrifugation at 12 000 × *g* for 10 min at 6 °C, 100 μl of the supernatant was assayed for luciferase activity. For GUS assays, 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 h. Then, 50 μl of the reaction mixture was diluted into 2 ml of 0.2 M Na_2CO_3 and the resulting fluorescence was measured in a Sequoia-Turner model 450 fluorometer which was adjusted to have a reading of 1000 units for 1 μM 4-methylumbelliferone. The normalized GUS activity represents the number of fluorescent units from an aliquot of extract that contained 2000 000 relative light units of luciferase activity.

Results

The promoter units necessary and sufficient for ABA response in two ABA-inducible GA-insensitive genes, *HVA1* and *HVA22*, have been defined (Shen and Ho, 1995; Shen *et al.*, 1996) and are shown in Figure 1. ABRC1 (49 bp) consists of an ACGT-box and a distal coupling element, CE1, while ABRC3 (22 bp) is composed of a similar ACGT-box and a proximal coupling element, CE3. The ACGT-boxes in these two complexes are fully exchangeable while the coupling elements are not (Shen *et al.*, 1996). The promoters of the GA-inducible but ABA-suppressible α-amylase genes are more complex, at least four regions necessary for GA induction have been identified (Gubler

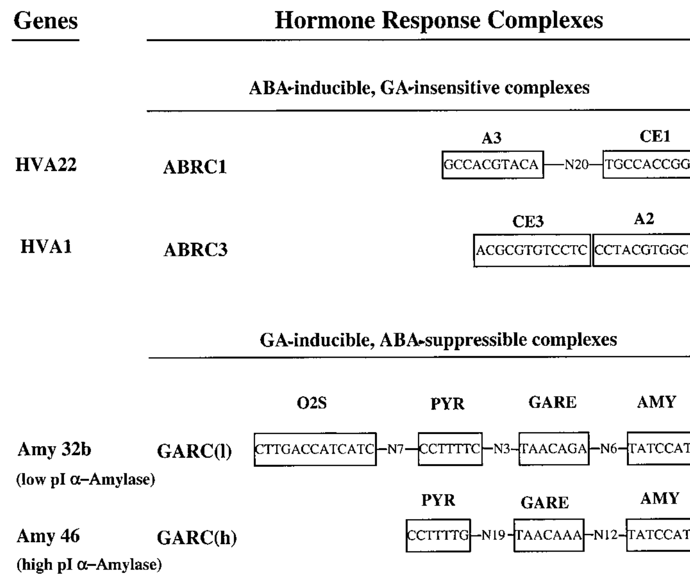

Figure 1. Important promoter regions in the abscisic acid response complexes (ABRCs) and gibberrellin response complexes (GARCs). A2 and A3, ACGT-core containing boxes (G-boxes or ABA response element [ABRE]); CE, coupling element; O2S, putative Opaque 2-binding sequence; Pyr, pyrimidine box; GARE, GA response element; Amy, amylase box (Box 1).

and Jacobsen, 1992; Lanahan *et al.*, 1992; Rogers and Rogers, 1992). Three of these regions are highly conserved among low- and high-pI α-amylase promoters. An additional important box (putative Opaque 2 binding sequence) is found only in the low-pI α-amylase promoters (Figure 1). The availability of these well-defined hormone-responsive complexes prompted us to study the signal transduction components mediating ABA induction and suppression of gene expression.

Internal control construct, Ub-Luc, is not affected by co-expression of effector constructs

Since the use of Ub-LUC as an internal control in this procedure is essential in minimizing the variations among particle bombardment, we have carried out an extensive analysis to make sure that the activity of ubiquitin/luciferase internal control plasmid is not affected by the presence of effectors such as 35S-abi1-1. As shown in Figure 2, the expression of Ub-LUC construct was not significantly affected by the presence of a wide range of effector constructs. Similar results were obtained with other effector constructs (data not shown). Therefore, we used the Ub-LUC construct as the internal control throughout the rest of this work.

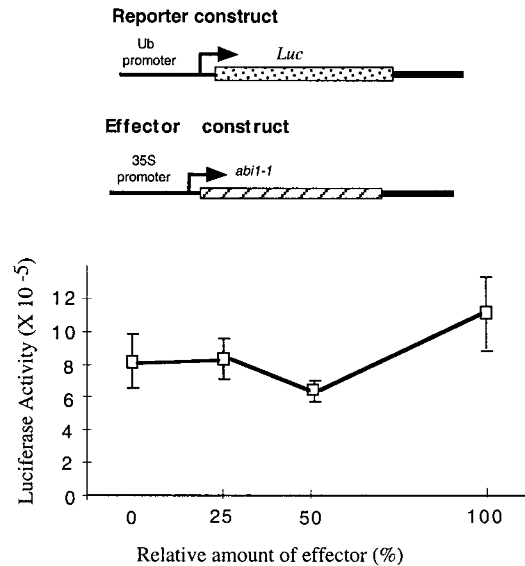

Figure 2. The expression of the internal control construct, a luciferase coding region linked to a constitutive promoter (Ub-LUC), is not affected by the coexpression of *abi1-1* effector gene. Ub-LUC DNA was co-bombarded into barley half-seeds along with the 35S-abi1-1 effector construct. The amount of Ub-LUC was always constant (1.14 μg per shot) whereas that of the effector varied with respect to Ub-LUC as shown in the X-axis.

Both the wild-type ABI1 and its dominant mutation form inhibit the ABA induction of ABRC

Among the genes that regulate the response to ABA, *ABI1* is interesting because it encodes a protein phosphatase 2C (Leung, *et al.*, 1994). A mutation of this gene, *abi1-1*, causes a reduction of phosphatase activity (Sheen, 1998; Gosti *et al.*, 1999) and this mutation is dominant negative in blocking ABA responses in *Arabidopsis* (Leung *et al.*, 1994; Meyer *et al.*, 1994). The coding sequence of the *ABI1* gene or its mutant *abi1-1* was fused to the non-ABA responsive CaMV 35S constitutive promoter, generating effector constructs, 35S-ABI1 and 35S-abil-1. The effect of these two constructs on ABA response was then studied by using two ABA-inducible reporter constructs, ABRC1-GUS and ABRC3-GUS. As shown in Figure 3, coexpression of 35S-abi1-1 had a very strong negative effect on the ABA induction of both promoters. When the relative amount of effector was 2% of the reporter construct, the ABA induction of ABRC1 decreased from 25-fold (no effector) to only 7-fold (Figure 3A), with the absolute level of the GUS activity dropping to 28% of the control (data not shown). At the same level, the effect of the 35S-ABI1 was negligible. However, increasing amounts of 35S-ABI1 had a readily observable effect on ABA induction of ABRC1 (Figure 3A). Although both wild-type and mutant forms of ABI1 are effective in blocking the ABA induction of gene expression, it is estimated that it takes 10 times more 35S-ABI1 than 35S-abi1-1 to achieve the same level of inhibition of ABA up-regulation of gene expression (Figure 3A).

ABA induction of ABRC3 was also inhibited by the constitutive expression of *ABI1* or *abi1-1*. However, compared with ABRC1, the pattern of suppression of ABRC3-GUS expression by the presence of mutant ABI1 was more moderate. When the relative amount of effector was 2% of the reporter construct, the effect of 35S-abi1-1 on ABRC3 was minimal (Figure 3B). However, increasing amounts of effector construct drastically inhibited the ABA induction of ABRC3. The effect of 35S-ABI1 on the ABA induction of ABRC3-GUS was comparable to that observed for ABRC1-GUS.

Mutant ABI1 does not affect the ABA suppression pathway

In barley aleurone cells, ABA induces the expression of late embryogenesis abundant (*LEA*) genes while suppresses the expression of germination specific genes such as those encoding α-amylases. The effect of the mutant ABI1 on these two pathways was compared in Figure 4. Co-bombardment of 35S-abi1-1 resulted in a decrease of the ABA induction of ABRC1-GUS to only 4-fold, compared to 46-fold obtained in the absence of effector construct. Similarly, mutant ABI1 also inhibited ABA induction of ABRC3; only a 4-fold ABA induction was obtained in the presence of the 35S-abi1-1 while the ABA induction was as high as 25-fold in the absence of effector construct (Figure 4A). These results are qualitatively consistent with those presented in Figure 3.

The specificity of the *ABI1* or *abi1-1* expression on ABA induction was confirmed by studying the effect of the coexpression of another protein phosphatase on the ABA induction of ABRC3-GUS. The coding sequence of the maize *ZmPP1* gene, which encodes a type 1 protein phosphatase (Smith and Walker, 1991), was linked to the *35S* promoter and used as an effector construct. As shown in Figure 4B, coexpression of ZmPP1 had little effect on either ABA induction or the level of GUS activity obtained from the reporter construct ABRC3-GUS.

The effect of the mutant ABI1 protein on the expression of the reporter construct GARC(l)-GUS was also studied to address two questions: (1) does the mutant ABI1 inhibit GA induction of the α-amylase reporter construct? and (2) does the mutant ABI1 have any effect on the ABA suppression of GA-induced α-amylase expression? In the absence of effector construct, GA treatment resulted in 131-fold induction of GARC(l)-GUS. Co-expression of 35S-abi1-1 did not have any effect on either GA induction or the level of GUS activity obtained from the reporter construct (Figure 4C). In addition, 35S-abi1-1 did not appear to have any effect on the ABA-suppression of GA-induced expression of GARC(l)-GUS (Figure 4C). Abscisic acid still effectively repressed the GA induction of α-amylase even in the presence of mutant ABI1. Similar results were obtained when the promoter of a high-pI α-amylase gene, *Amy46*, was used in the reporter construct (data not shown). Therefore, the effect of the mutant ABI1 appeared to be specific for the ABA induction pathway.

VP1 promotes the ABA induction pathway but also inhibits the ABA suppression pathway

In the aleurone system, co-expression of VP1 enhanced the ABA induction of ABRC3. Results of

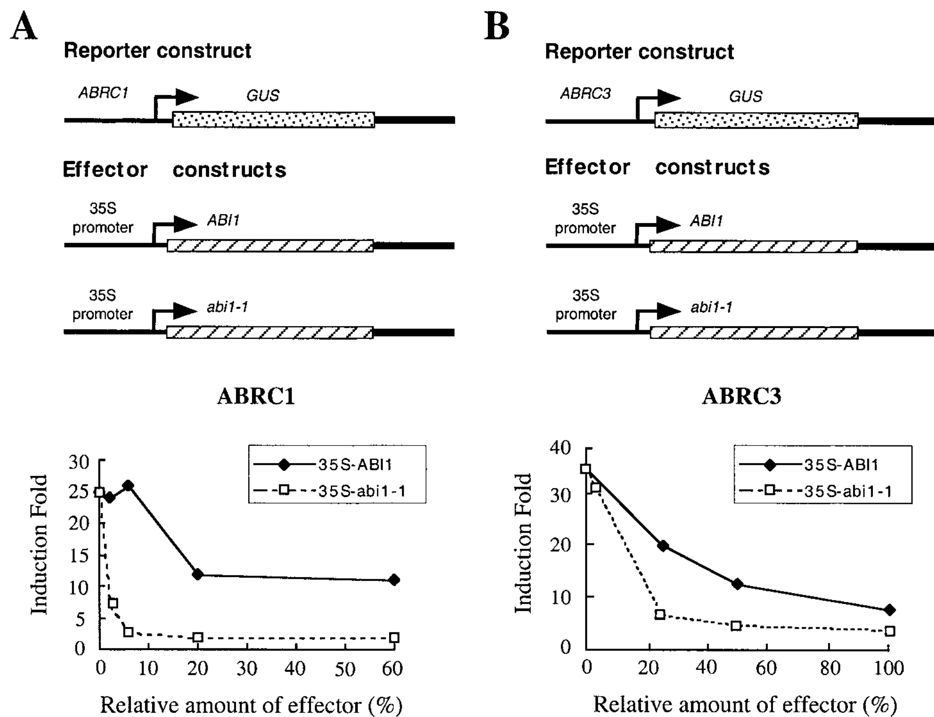

Figure 3. Both ABI1 and its dominant mutant inhibit the ABA induction of ABRC1 and ABRC3. A. The 35S-ABI1 or 35S-abi1-1 effector construct was co-bombarded into barley half-seeds along with the reporter construct (ABRC1-GUS) and the internal control construct (Ub-LUC). The amount of reporter and control plasmid DNA was always constant (1.14 μg per shot), whereas that of the effector varied with respect to the reporter as shown on the x axis. Transformed half-seeds were incubated for 24 h with 20 μM ABA. Induction fold obtained with each treatment was plotted. The solid line represents the effect of the wild type ABI1 and the dashed line depicts that of the mutant ABI1. B. All conditions were the same as in A except that the reporter construct was ABRC3-GUS.

a typical experiment are shown in Figure 5. In the absence of hormones, co-expression of 35S-VP1 promoted a small yet consistent induction (3-fold) of ABRC3-GUS, compared to 22-fold obtained with the ABA treatment (Figure 5A). Interestingly, in the presence of ABA, co-expression of both *VP1* and *abi1-1* blocked the ABRC3 induction in a way similar to that obtained with the co-expression of *abi1-1* alone (Figure 5A).

VP1 also had a suppressive effect on the GA induction of gene expression. In the absence of VP1, GA treatment led to a 25-fold induction of GARC-GUS. Co-expression of 35S-VP1 drastically reduced the GA induction of this reporter construct to only 2-fold (Figure 5B).

PKABA1 is specifically involved in the ABA suppression of gene expression

The role of the ABA-inducible protein kinase, PKABA1, in mediating the ABA suppression of gene expression was studied by co-expressing 35S-PKABA1 with a high-pI α-amylase reporter construct (Figure 6A). In the absence of effector, the expression of the GARC(h)-GUS reporter construct was highly responsive to GA_3. However, the GA induction of GARC(h)-GUS was suppressed by increasing amounts of PKABA1 (data not shown). These observations are similar to the effect of PKABA1 on another α-amylase promoter, GARC(l) reported before (Gomez-Cadenas *et al.*, 1999). The effect of several CDPKs on the suppression of GA induction of GARC was also tested (Figure 6A). When equal amounts of effector and reporter constructs were introduced into aleurone cells, 35S-PKABA1 blocked the response of GARC(h) to GA while none of the other protein kinases driven by the same constitutive promoter had any significant effect (Figure 6A).

The role of protein kinases on the ABA induction of gene expression was also studied by co-bombardment of different CDPKs and PKABA1 effector constructs with ABRC3-GUS (Figure 6B). Our results showed that co-expression of different protein kinases (using equal amount of effector and reporter

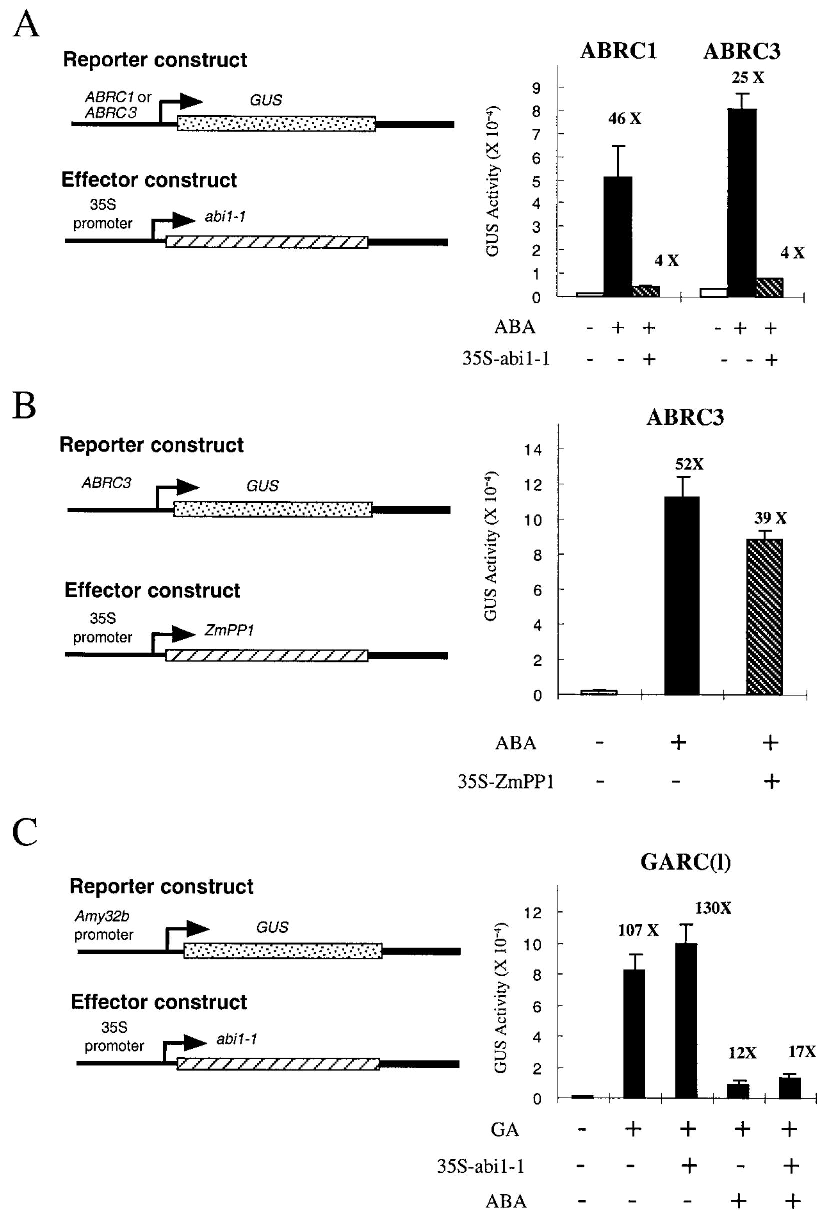

Figure 4. Mutant ABI1 is a negative regulator specific for the ABA induction pathway. A. mutant ABI1 has specific inhibitory effect on both ABRC1 and ABRC3 in response to ABA treatment. The reporter construct, ABRC1-GUS or ABRC3-GUS, and the internal control, Ub-LUC, were co-bombarded into barley half-seeds either with or without the effector construct, 35S-abi1-1. Same amounts of effector and reporter constructs (1.14 μg per shot) were used. The numbers on the top of bars represent induction fold from each treatment. Transformed half-seeds were incubated for 24 h with or without 20 μM ABA. B. The ABA responsiveness of ABRC3 is not affected by ZmPP1, a type 1 protein phosphatase from maize. The test conditions were the same as in A except that the effector construct was 35S-ZmPP1. C. Mutant ABI1 has no effect on either the GA induction or the ABA suppression of α-amylase expression. The test conditions were the same as in A except that the reporter construct was GARC(l)-GUS. Transformed half-seeds were incubated for 24 h with or without 1 μM GA and/or 20 μM ABA.

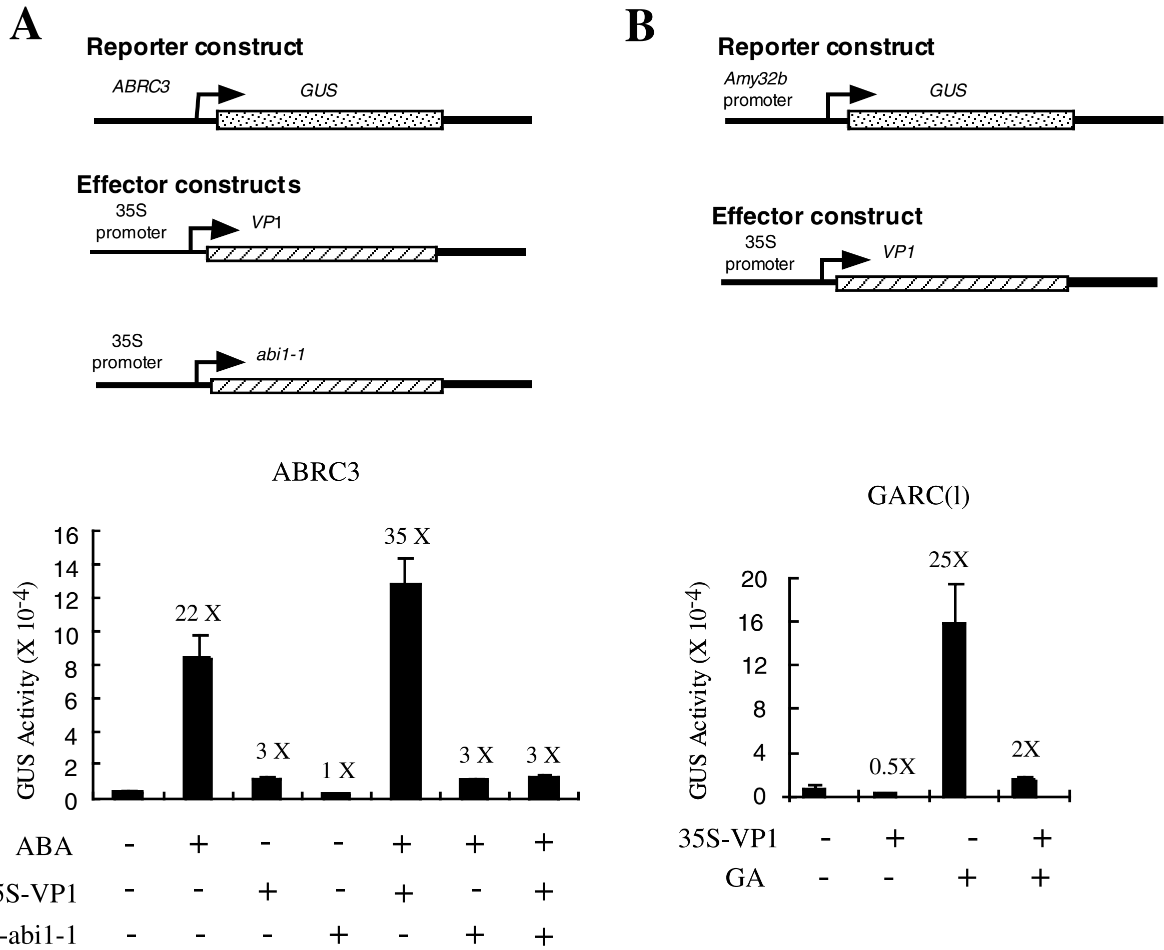

Figure 5. VP1 affects the hormonal response of ABA- and GA-responsive promoters. A. ABRC3 is responsive to VP1. The reporter construct, ABRC3-GUS, and the internal control, Ub-LUC, were co-bombarded into barley half-seeds either with or without effector constructs (35S-VP1 and 35S-abi1-1). Same amounts of effector and reporter constructs (1.14 μg per shot) were used. Transformed half-seeds were incubated for 24 h with or without 20 μM ABA. The numbers on the top of bars represent induction fold from each treatment. B. The response of GARC to GA is suppressed by VP1. The test conditions were the same as in A except that the reporter construct was GARC(l)-GUS. Transformed barley half-seeds were incubated for 24 h with or without 1 μM GA.

constructs) did not have any significant effect on the response of ABRC3 to ABA. However, CDPK1, CDPK1a and PKABA1 slightly increased the background expression of ABRC3 reporter construct in the absence of ABA (Figure 6B, open bars). However, neither CDPKci nor the null mutation of CDPK1, which lacks the nucleotide binding domain, had any effect on the basal levels of ABRC3 expression (Figure 6B). Similar results were obtained while using ABRC1 as a reporter construct (data not shown).

Discussion

The mode of action of ABA in barley aleurone layers is complex because this hormone not only induces the expression of late embryogenesis genes like *HVA1* and *HVA22*, but also suppresses GA-responsive genes such as those encoding α-amylases and proteases. Pharmacological approaches indicate that a phospholipase D activity is involved in the transduction of the ABA signal (Ritchie and Gilroy, 1998). The involvement of reversible protein phosphorylation has also been demonstrated by using protein phosphatase/kinase inhibitors (Heimovaara *et al.*, 1995; Kuo *et al.*, 1996).

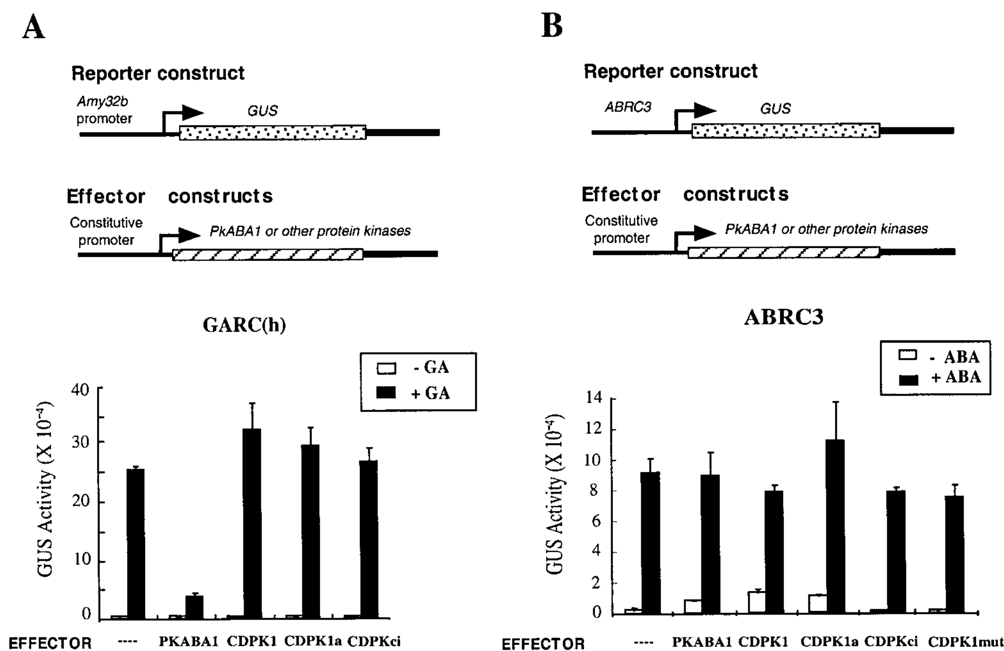

Figure 6. An ABA-induced protein kinase, PKABA1, suppresses GA induction of GARC. A. The reporter construct, GARC(h)-GUS, and the internal control, Ub-LUC, were co-bombarded into barley half-seeds either with or without effector constructs (shown on the x axis). Same amounts of effector and reporter constructs (1.14 μg per shot) were used. Transformed half-seeds were incubated for 24 h with or without 1 μM GA. B. The test conditions are the same as in A except that the reporter construct was ABRC3-GUS. Transformed half-seeds were incubated for 24 h with or without 20 μM ABA.

Following a different strategy, we have attempted to dissect the ABA signal transduction pathways by investigating the interactions among regulatory molecules known to mediate ABA action. Co-bombardment of genes encoding these regulatory molecules along with hormone responsive gene constructs mimics what has been observed in genetic analyses. For example, co-expression of *ABI1* or *abi1-1* leads to an inhibitory effect on the ABA induction of two different genes, *HVA1* and *HVA22*. Co-expression of *VP1* has a synergistic effect on ABA induction of *HVA1*, yet it also suppresses the GA induction of α-amylase expression, a phenomenon in line with the suggested role of VP1 in regulating seed dormancy/germination (Hattori *et al.*, 1992). Furthermore, an ABA-induced protein kinase, PKABA1, specifically mediates the ABA suppression of α-amylase expression while several other protein kinases, i.e. various CDPKs, have no effect on this process. Therefore, the co-bombardment strategy followed in this work offers an opportunity to dissect ABA signal transduction pathways. Similar strategies have been used before to investigate the involvement of several intermediates in hormone signal transduction (Gubler *et al.*, 1995; Sheen, 1996, 1998; Shen *et al.*, 1996; Abe *et al.*, 1997; Kovtun *et al.*, 1998; Gomez-Cadenas *et al.*, 1999).

It has been shown that ABA exerts a tight regulation on the expression of *HVA1* and *HVA22* (Hong *et al.*, 1992; Shen *et al.*, 1993), and a similar phenomenon is observed in this work using reporter constructs containing promoters from these genes linked to the GUS-coding region. The constitutive expression of either *ABI1* or the *abi1-1* mutant suppresses the ABA induction of *HVA1* and *HVA22*. However, the inhibitory effect of the *abi1-1* gene product is significantly stronger than that of the wild type ABI1. Recent work by Gosti *et al.* (1999) has suggested that the PP2C encoded by *ABI1* is a negative regulator of ABA signaling in *Arabidopsis*. These authors have shown that several mutations reducing the PP2C activity lead to enhanced responses to ABA. However, the dominant *abi1-1* mutation also causes a significant reduction of PP2C activity. It has been hypothesized that the *abi1-1* mutation would either form a poison complex with the putative substrate or its activity would not be regulated by ABA the same way as the wild-type ABI1 (Gosti *et al.*, 1999). Our results further support the genetic data of Gosti *et al.* (1999) indicating that ABI1 has a role as a negative regulator in ABA

signal transduction. Specificity of ABI1 in inhibiting ABA induction is suggested by the experiment shown in Figure 4B. Expression of another protein phosphatase, maize PP1, driven by the same constitutive promoter has little effect on inhibiting ABA induction. Although PP2C sequences have been cloned in maize (GenBank accession number U81960NID) and rice (AF075603NID), whether these PP2C function like the *Arabidopsis* ABI1 awaits further studies.

ABA is also known to down-regulate the expression of genes involved in seed germination, seedling growth and photosynthesis. The involvement of PP2C in mediating the ABA suppression of a photosynthetic gene promoter has been previously reported (Sheen, 1998). This could point to ABI1 having a role in the ABA suppression of α-amylase gene expression. However, data in Figure 4C indicate that in barley aleurone layers, mutant ABI1 does not affect either the GA induction of the hydrolytic enzymes or the ABA suppression of this process. Therefore, the ABA induction and suppression appear to follow two separate pathways with the former being inhibited and the latter unaffected by ABI1. The lack of effect of mutant ABI1 on the expression of α-amylase, which plays an important role in seedling growth, is also in agreement with the observation that the *abi1-1* mutant in *Arabidopsis* does not lead to precocious germination (vivipary).

In contrast to *ABI1*, mutation of the maize *VP1* gene renders maize seeds insensitive to ABA and lead to precocious germination (McCarty *et al.*, 1989). Hence, we compared the effect of VP1 on the expression of *HVA1* (ABRC3) with that on the α-amylase gene (GARC). Our results show that VP1 promotes ABA induction of *HVA1*, but also suppresses GA-induced α-amylase expression (Figure 5). This is consistent with the report that VP1 is a transactivator of the wheat ABA-inducible *Em* gene promoter and suppressor of an α-amylase gene promoter (Hoecker *et al.*, 1995). These data support the hypothesis that VP1, acting in seed developmental programs, interact with the ABA signal transduction leading to the induction of embryogenesis related genes and also to the suppression of germination-specific genes. However, it is not yet clear whether ABI1 and VP1 act in the same or in overlapping signaling pathways in seeds (Leung and Giraudat, 1998). Our experiments indicate that mutant ABI1 and VP1 interact, either directly or indirectly, in mediating ABA up-regulated gene expression. When both *VP1* and *abi1-1* were expressed in the barley aleurone tissue at the same time, ABA induction of ABRC3 was dramatically inhibited,

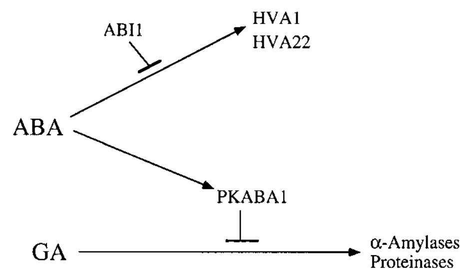

Figure 7. A schematic model of ABA signal transduction pathways showing separate pathways for induction (→) and suppression (–|) of gene expression.

with the expression level similar to that obtained with mutant ABI1 alone (Figure 5). Therefore, the mutant ABI1 inhibitory effect is able to overcome the transactivation effect of VP1 on ABA induction as well as the synergism of ABA and VP1. Recently, VP1 has been shown to be part of the transcription complex leading to the expression of wheat *Em* gene (Schultz *et al.*, 1998), we speculate that mutant ABI1 can destabilize a similar complex binding to ABRC3 either by acting directly on VP1 or on other proteins of this complex.

The role of PKABA1 acting as an intermediary in the down-regulation of low-pI α-amylases has been previously studied (Gomez-Cadenas *et al.*, 1999). Here, we observe the same effect of PKABA1 on a different group of α-amylase genes (Figure 6A) which allows us to propose a wider effect for PKABA1 as a central regulator of the ABA suppression of the hydrolytic enzyme expression. The fact that co-expression of another group of protein kinases, i.e. CDPKs, fails to inhibit the α-amylase expression, strongly suggests a specific role for PKABA1 in this suppressory process (Figure 6A). Recently, another protein kinase, AAPK, has been shown to be essential for the ABA-induced stomatal closure (Li et al., 2000). However, AAPK has no effect in the aleurone cells (data not shown). We have also tested the effect of several other protein kinases in this work. Data presented in Figure 6B indicate that none of the protein kinases tested in this work alter the ABA induction of *HVA1*. However, CDPK1, CDPK1a and PKABA1 do have a relative small, but noticeable, effect on the basal expression levels of the HVA1 reporter construct in the absence of hormones. This effect of CDPK1 and CDPK1a is much more moderate than observations made in maize leaf protoplasts where both protein kinases fully transactivate the expression of the same *HVA1* gene (Sheen, 1996). These differences between barley aleurone and maize leaf cells could also reflect

the differential effect of ABA treatment on Ca^{2+} levels, i.e., while ABA promotes an increase in the levels of Ca^{2+} in vegetative tissues (Wu *et al.*, 1997), it reduces the intracellular Ca^{2+} concentration in aleurone layers (Gilroy, 1996). Therefore, we suggest that, in contrast to leaf cells, the signal transduction pathway leading to ABA induction of *HVA1* and *HVA22* in the aleurone cells could be Ca^{2+}-independent. The existence of an alternative pathway or the co-existence of parallel pathways in different cell types needs to be further explored.

As summarized in Figure 7, we have demonstrated the existence of two separate signal transduction pathways leading to the induction or suppression of gene expression mediated by ABA in the barley aleurone cells. The inductive pathway is mediated by the ABI1 protein phosphatase 2C while the suppressive pathway is modulated through the protein kinase PKABA1. It is not clear whether these two pathways share some common steps, however, both of them can be modulated by the presence of VP1. It has also been reported that both the ABA induction and suppression of gene expression are mediated by phospholipase D (Richie and Gilroy, 1998). The molecular mechanism underlying the interactions among these regulatory molecules is the subject of future investigations.

Acknowledgments

We thank Dr John Rogers for providing the *Amy64* minimal promoter and the high-pI α-amylase reporter constructs, Dr Donald McCarty for the 35S-VP1 construct, Dr John Walker for the *ZmPP1* cDNA clone, and Drs Jeff Harper and Bimei Hong for some of the CDPK clones. Finally, we are grateful to Drs Danniel Kohl and Craig Pikkard for their critical reading of the manuscript. This work was supported by grants from NSF and USDA to T-HDH, and a fellowship from Ministero de Educacion y Cultura, Spain to AG-C.

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