Protein Binding to the Absciscic Acid–Responsive Element Is Independent of VIVIPAROUS1 in Vivo

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The plant hormone abscisic acid and the transcriptional activator VIVIPAROUS1 have a synergistic effect on transcription during embryo development. An abscisic acid–responsive element (ABRE) mediates induction by abscisic acid and VIVIPAROUS1, but the mechanism involved has not been determined. In this study, we explore the interaction between abscisic acid and VIVIPAROUS1 and its effect on the ABRE from the maize *rab28* gene. In transient transformation experiments, abscisic acid stimulated transcription via several elements, whereas activation by VIVIPAROUS1 was mediated exclusively through the ABRE. In vivo footprinting showed only minor differences in binding to the ABRE between wild-type and VIVIPAROUS1-deficient embryos, suggesting that VIVIPAROUS1 stimulates transcription through the ABRE without major changes in protein–DNA interactions. A factor that bound to the ABRE in electrophoretic mobility shift assays was present at the same developmental stages as *rab28* mRNA and had binding characteristics similar to those observed by in vivo footprinting. This suggests that the factor binds to the ABRE in the *rab28* promoter in vivo. We discuss the constraints that our results put on the possible mechanism for action of VIVIPAROUS1 in vivo.

INTRODUCTION

The plant hormone abscisic acid (ABA) is a regulator of gene expression during embryo maturation (reviewed in Giraudat et al., 1994). ABA also plays a critical role in the response of vegetative tissue to stresses, such as drought, cold, and high salt concentrations (reviewed in Ingram and Bartels, 1996). Studies of the promoters of ABA-responsive genes have revealed an ABA-responsive element (ABRE) with the core sequence ACGT capable of mediating ABA-inducible transcription (Marcotte et al., 1989; Mundy et al., 1990). The ABRE is homologous to the G-box (Izawa et al., 1993), which is implicated in the response to various environmental stimuli, such as light (Giuliano et al., 1988), UV light (Weisshaar et al., 1991), and anaerobiosis (McKendree and Ferl, 1992). A variety of basic leucine zipper (bZIP) transcription factors binds to the element in vitro, but none has yet been shown unambiguously to mediate induction by ABA in vivo (Lu et al., 1996).

The bases flanking the ACGT core of the ABRE play a crucial role in protein binding in vitro (Izawa et al., 1993) and affect the function in vivo (Salinas et al., 1992; Busk et al., 1997). However, the available data favor a model in which

the synergistic action of the ABRE together with other elements is necessary for the specificity of ABA-inducible promoters (Shen and Ho, 1995; Shen et al., 1996).

Genetic studies with maize have shown that mutants in the seed-specific developmental activator VIVIPAROUS1 (VP1) have reduced sensitivity to ABA and do not express several ABA-inducible genes (reviewed in McCarty, 1995). In addition, ectopic expression of the ABA-insensitive VP1 homolog ABI3 from Arabidopsis results in the induction of otherwise seed-specific genes in response to ABA in vegetative tissues (Parcy et al., 1994).

Expression of the ABA-inducible genes *Em* and *rab28* in the maize embryo is controlled by VP1 (McCarty et al., 1991; Pla et al., 1991). However, VP1 is present at earlier developmental stages than are *rab28* and *Em* mRNA. Therefore, other developmental cues are necessary for the expression of these genes. Transcription of the *rab28* and *Em* genes is low or absent in VP1-deficient embryos but can be induced by high concentrations of exogenously applied ABA (Pla et al., 1991; Williamson and Scandalios, 1992). Promoter analysis has shown that there is a synergistic effect of ABA and VP1 on the ABREs of *Em* (Hattori et al., 1995; Vasil et al., 1995). This effect mimics the developmental regulation of *Em*. The ABRE could also be important for the regulation of *rab28*, because a sequence of 134 bp with a putative ABRE is sufficient for induction by ABA of the *rab28* promoter (Pla et al., 1993). Therefore, the ABRE is central for control of

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Figure 1. The Putative ABA-Responsive Sequences in the Minimal *rab28* Promoter Inducible by ABA.

The putative elements are enclosed in boxes. Numbering is relative to the transcription start point.

gene expression in the embryo. An understanding of how VP1 and ABA stimulate transcription through the ABRE will give important information about the regulation of embryogenesis.

In vitro, VP1 stimulates binding of the bZIP transcription factor EmBP-1 to the ABRE, probably by a transient or unstable interaction with the DNA (Hill et al., 1996). However, VP1 enhances binding in vitro of different classes of plant and animal transcription factors to their target sites in an apparently unspecific manner. Therefore, it remains to be determined how VP1 stimulates transcription through the ABRE in vivo.

We have used the *rab28* promoter as a model system to understand the stimulation of transcription by VP1 and ABA via the ABRE. VP1 and ABA induced transcription through the two ABREs of the *rab28* promoter in transient transformation assays in the same way as has been shown for *Em*. In vivo footprinting in wild-type and VP1-deficient embryos showed that protein binding to the ABREs is independent of VP1. However, there was a minor enhancement of the footprint in the presence of VP1. These results suggest that VP1 activates transcription via the ABRE, mainly by a mechanism that does not involve changes in protein-DNA interactions. The activity of an ABRE binding factor (ABF) from nuclear extracts correlated with the developmental expression of *rab28*. This factor could be binding to the ABREs of *rab28* in vivo and mediating induction by VP1 and ABA.

RESULTS

VP1- and ABA-Inducible Elements in the *rab28* Promoter

The sequence from 60 to 193 bp upstream of the transcription start of the *rab28* gene is sufficient to confer inducibility by ABA on a cauliflower mosaic virus 35S -90 to +35 promoter in rice protoplasts (Pla et al., 1993). Four putative ABA-inducible elements present in this sequence are depicted in Figure 1. ABRE A binds nuclear proteins from embryos and leaves and was postulated to be involved in the ABA response of *rab28* (Pla et al., 1993). ABRE B is another putative ABRE in the *rab28* promoter. In addition, there is a

sequence with similarity (seven of 12 bases) to the GRA element from the maize *rab17* promoter (Busk et al., 1997) and a sequence with 10 of 12 bases identical to the coupling element CE3 from the *HVA1* promoter from barley (Shen et al., 1996).

To define the function of these elements, we performed transient transformation of maize embryonic callus with the *rab28* promoter construct pMP130 (Pla et al., 1993). The inducibility by ABA of the wild-type construct and mutants of each of the two ABREs (ABRE A⁻ and ABRE B⁻), the double mutant (ABRE A⁻B⁻), and a GRA mutant (GRA⁻) and a CE3 mutant (CE3⁻) was tested, and the results are presented in Figure 2. In callus, the ABRE A⁻ construct conferred weak induction by ABA compared with that of the wild type, showing that ABRE A is functional. In contrast, the ABRE B⁻ construct had almost the same inducibility as the wild type did. Disruption of both of the ABREs (ABRE A⁻B⁻) abolished the activity of the promoter. The GRA⁻ and CE3⁻ constructs had lowered inducibility, but it was not as low as that of ABRE A⁻.

The developmental activation of *rab28* depends on VP1 but can be partially overcome by exogenous ABA (Pla et al., 1991). We wanted to test whether VP1 enhances transcription through the four elements described above. Cotransformation of a VP1 expression plasmid with the *rab28* promoter stimulated transcription (Figure 2). In addition, as shown in Table 1, induction by ABA and VP1 was cooperative.

Figure 2. Activity of the *rab28* Promoter Constructs in Transient Transformation of Maize Callus.

The values represent the mean of three experiments. Standard deviations are represented by error bars. Relative β -glucuronidase (GUS) activity was calculated as described in Methods. WT, wild type; A⁻, ABRE A⁻; B⁻, ABRE B⁻; A⁻B⁻, ABRE A⁻B⁻; AB, the plasmid with ABRE A and ABRE B in front of a 35S promoter.

Table 1. Fold Induction by VP1^a

Construct ^b	No ABA	ABA (100 μ M)
WT	2.6	4.1
A ⁻	0.6	2.3
B ⁻	0.6	2.3
GRA ⁻	2.6	7.5
CE3 ⁻	1.3	5.8

^aCalculated from the data shown in Figure 2 by dividing the activity of the construct when cotransformed with VP1 by the activity of the construct without VP1.

^bWT, wild type; A⁻, ABRE A⁻; B⁻, ABRE B⁻.

In the presence of VP1, the activities of ABRE A⁻ and ABRE B⁻ were much lower than the activity of the wild-type promoter, suggesting that the ABREs mediated induction by VP1 (Figure 2). The two mutated constructs showed the same reduction in inducibility by VP1, suggesting that VP1 regulated transcription through ABRE A and ABRE B by using the same mechanism (Table 1). Curiously, VP1 repressed transcription of the ABRE A⁻ and ABRE B⁻ constructs in the absence of ABA. A construct with ABRE A and ABRE B in front of the 35S promoter demonstrated that the sequence of the ABREs was sufficient for induction by ABA and VP1, although the level of expression was much less than that of the wild-type construct (Figure 2). Without ABA, CE3⁻ was less inducible by VP1 than was the wild type, but twice as inducible as ABRE A⁻ and ABRE B⁻ (Table 1). In the presence of ABA, GRA⁻ and CE3⁻ had the same fold of induction by VP1 as did the wild-type construct.

In Vivo Footprinting of the *rab28* Promoter in Wild-Type Embryos

To investigate whether the results obtained by expression of the *rab28* promoter in callus were relevant for the developmental activation of *rab28*, we performed in vivo footprinting of the *rab28* promoter in embryos 28 days after pollination (DAP). In these embryos, the *rab28* gene is developmentally expressed (Pla et al., 1991; Niogret et al., 1996).

Figures 3A and 3B show that there was a footprint on ABRE A, with protection of the guanines in the central sequence (CACGTG) and hypersensitivity of the flanking guanines. The protections were almost complete, showing that there was strong binding to the element. Qualitatively, the same footprint was observed on ABRE B; however, on this element, the central bases were less protected, indicating a lower affinity for the factor. The weaker footprint on ABRE B is in accordance with the activity of the ABRE B⁻ construct in transient transformation. There were no changes in the footprints on the two ABREs in excised embryos incubated for 24 hr with ABA, whereas the footprint on ABRE B

Figure 3. In Vivo Footprinting of the *rab28* Promoter in 28-DAP Wild-Type Embryos.

In vitro-methylated DNA (lane N) compared with DNA from 28-DAP embryos treated with dimethylsulfate (DMS) within 15 min after excision from the cob (lane C) and DNA from embryos treated with DMS after 24 hr of incubation with 100 μ M ABA (lane A) or water (lane W).

(A) The nontranscribed strand from positions -91 to -155.

(B) The transcribed strand from positions -93 to -156.

The scanning profile of each lane is shown next to the sequence ladder. The extent of the elements mentioned in Figure 1 is indicated with bars. Filled arrows, reduced reactivity (protection) in vivo; open arrows, enhanced reactivity (hypersensitivity) in vivo.

became weaker in embryos incubated without ABA (Figures 3A and 3B).

We detected protein–DNA interactions on the two non-ABRE elements. CE3 had two protections and a clear hypersensitivity, whereas the footprint on the GRA was weak (Figure 3A). Thus, the *in vivo* footprinting showed that all four elements studied by transient transformation bound proteins *in vivo*.

In Vivo Footprinting in VP1-Deficient Embryos

By *in vivo* footprinting of the *vp1* mutant, we were able to determine whether VP1 induces transcription of *rab28* by enhancing protein binding to the two ABREs in the promoter. Figures 4A and 4B show that there was a clear footprint on ABRE A in VP1-deficient embryos. The footprint was slightly weaker than that in wild-type embryos, but the difference was at the limit of detection. This shows that VP1 had only a minor effect on protein binding to ABRE A *in vivo*.

On ABRE B, the footprint in the *vp1* mutant was weaker than that in the wild-type embryos (Figures 4A and 4B). The difference between the wild type and *vp1* was clearest on the protected residue (–103) on the lower strand, which was more protected in the wild type than in the *vp1* mutant (Figures 3A and 4A).

In excised VP1-deficient embryos incubated with ABA, the protein–DNA interactions on ABRE A and ABRE B were weaker. Incubation of excised embryos in water clearly reduced the footprint on ABRE A, and the footprint on ABRE B almost disappeared (Figures 4A and 4B, lanes A and W). This was different from the wild type and shows that VP1 deficiency leads to changes in the protein–DNA interactions on the ABREs in excised embryos.

Apart from the footprints, there are some differences in the sequence ladder of naked DNA (Figures 3A and 4A, lanes N) between the wild type and the *vp1* mutant. These differences are found in the GRA and CE3 regions and are due to polymorphisms between the lines. The polymorphisms do not affect the regulation of the *rab28* gene (Pla et al., 1991).

A Developmentally Regulated ABF Binds to the Two ABREs in the *rab28* Promoter with Different Affinity

The different extent of protein binding to ABRE A and ABRE B *in vivo* could be caused by different affinities for an ABF or could be an effect of chromatin structure. Therefore, we performed electrophoretic mobility shift assays with nuclear extracts from embryos and oligonucleotides with the sequence of ABRE A or ABRE B.

As shown in Figure 5A, a retarded complex was formed when nuclear extracts were incubated with ABRE A. The binding could be competed by 100-fold excess of unlabeled ABRE A but not with an ABRE A[–] oligonucleotide, showing

Figure 4. In Vivo Footprinting of the *rab28* Promoter in 28-DAP *vp1* Embryos.

(A) The nontranscribed strand from positions –91 to –155.

(B) The transcribed strand from positions –93 to –156.

All symbols are as given in the legend to Figure 3.

that binding was specific. Unlabeled ABRE B competed less than did ABRE A, indicating that ABRE B had a lower affinity for the ABF. When ABRE B was used as a probe, the retarded complex was observed only upon long exposure (Figure 5B). Competition of this complex confirmed that an ABF from embryos bound better to ABRE A than it did to ABRE B. Extracts from VP1-deficient embryos formed the same retarded complex (P.K. Busk and M. Pagès, unpublished data).

As shown in Figures 6A and 6B, the ABF was present in nuclear extracts from 25- to 60-DAP embryos in which the *rab28* gene was active. Only a weak band with the same mobility was observed in 14-DAP embryos in which *rab28* was not transcribed (Figure 6B). However, extracts from 14-DAP embryos formed complexes with different mobilities, suggesting that other factors with a similar specificity are present in young embryos. The activity of the ABF was highest in 25-DAP embryos and decreased when germination was induced by incubating the embryos in water. However, high activity of the ABF was observed in extracts from embryos incubated with ABA. These results show that there is a positive correlation between expression of *rab28* and the activity of an ABF.

DISCUSSION

Transactivation by VP1 through ABRE

VP1 potentiates the response to ABA in the maize embryo but is not an integrated part of the ABA signaling pathway (Hattori et al., 1992; Vasil et al., 1995). Embryogenesis and ABA induce the *catalase1* gene independently of VP1, showing that VP1 does not activate all ABA-inducible genes (Williamson and Scandalios, 1992). Hill et al. (1996) proposed that VP1 only regulates embryo-specific genes. However, expression of *rab28* is clearly lower in the *vp1* mutant, although the gene is not embryo specific (Pla et al., 1991). In fact, both *rab28* and the embryo-specific maize *Em* gene are dependent on VP1 for expression during normal seed development, but they can be induced by ABA in excised VP1-deficient embryos (Pla et al., 1991; Williamson and Scandalios, 1992).

VP1 mediates activation through the ABREs of the promoters of the *Em* genes from cereals (Hattori et al., 1995; Vasil et al., 1995) in the same way as we show here for *rab28*. Stimulation of transcription by VP1 of the *Em* gene promoters depends on an acidic activation domain, indicating that VP1 contacts the transcriptional machinery (McCarty et al., 1991; Hattori et al., 1995). In addition, VP1 enhances binding of EmBP-1 to the ABRE in vitro (Hill et al., 1996). A domain of VP1 rich in basic amino acids is necessary for this function and for induction of *Em* transcription. Here, we show that VP1 had little influence on protein binding to the ABREs of the *rab28* promoter, as detected by in vivo footprinting.

Figure 5. A Nuclear Protein Factor from Embryos Binds Specifically to the ABREs of the *rab28* Promoter.

(A) Electrophoretic mobility shift assay with probe A (overnight exposure).

(B) Electrophoretic mobility shift assay with probe B (5-day exposure). Nuclear extracts (45 μ g) from 37-DAP embryos were preincubated with a 100- or 1000-time excess of unlabeled competitor, as shown above each lane. (-), probe without nuclear extract/competitor; (+), probe with the nuclear extract. The retarded complex mentioned in the text is indicated by an arrow. A, ABRE A; B, ABRE B; A⁻, ABRE A⁻; B⁻, ABRE B⁻.

There was strong binding to the ABREs in the absence of VP1. This observation excludes the possibility that VP1 is necessary for opening the chromatin structure of ABA-induced genes. Interestingly, EmBP-1 binds to the ABRE on nucleosomes and interacts with histone H1, suggesting that

Figure 6. Activity of the ABF during Development.

(A) Electrophoretic mobility shift assay with probe A and nuclear extracts from different developmental stages. The age of the embryos in days is indicated above each lane. H₂O, extract from embryos of mature seeds incubated in water for the time indicated; ABA, extract

EmBP-1 or related proteins could be responsible for providing and maintaining an open chromatin structure on ABRE-containing promoters (Niu et al., 1996; Schultz et al., 1996).

Although it is possible that VP1 contacts the DNA, our data make it highly unlikely that VP1 binds by itself to the ABRE, which is in agreement with previous conclusions (McCarty et al., 1991). In vitro, VP1 interacts transiently and unspecifically with DNA (Hill et al., 1996). Proteins with these characteristics are difficult to detect by dimethyl sulfate (DMS) footprinting (Rigaud et al., 1991). Therefore, it cannot be excluded that VP1 binds to the *rab28* promoter, although we have been unable to attribute a footprint to binding of VP1. Suzuki et al. (1997) showed that the B3 domain of VP1 binds specifically to the SPH element of the *C1* promoter. The B3 domain is not necessary for activation through the ABRE (Vasil et al., 1995) and does not bind specifically to this element. Therefore, the results obtained for the interaction of VP1 with the SPH element do not account for activation through the ABRE (Suzuki et al., 1997).

The slight weakening of the footprint in the *vp1* mutant points to VP1 as enhancing binding to the ABRE, as has been shown in vitro (Hill et al., 1996). However, the weakened footprint could be caused by vivipary and the desiccation deficiency of the mutant without being related to the mechanism of VP1 action via the ABRE.

It has been proposed that VP1 binds indirectly to the ABRE by protein-protein interactions with the ABF or an associated factor (Hattori et al., 1995; Vasil et al., 1995; Suzuki et al., 1997). In this scenario, VP1 functions as a coactivator that binds to a preformed promoter complex and stimulates transcription by means of its acidic activator domain (Hill et al., 1996). Induction by a coactivator would be expected to result in the same protein-DNA interactions in the wild-type and VP1-deficient embryos, in agreement with the in vivo footprinting presented here.

The different effects of VP1 on protein binding to the ABRE observed in vivo and in vitro must be due to the different binding conditions. In the in vitro experiment, the effect of VP1 was assayed on free DNA and protein (Hill et al., 1996). However, in vivo, there is near saturating binding to the ABRE in the absence of VP1. Proteins other than VP1 can enhance binding to the ABRE and could create a favorable environment for occupancy of the element in vivo (Schultz et al., 1996; P.K. Busk, M. Figueras, and M. Pagès, unpublished data). In excised embryos, especially without

from embryos of mature seeds incubated in 100 μ M ABA for the time indicated. All binding reactions contained 45 μ g of nuclear protein, except for 14-DAP embryos, for which 5 μ g of nuclear extract and 45 μ g of BSA were used. The retarded complex mentioned in the text is indicated by an arrow.

(B) The same as given in **(A)** but with 5 μ g of nuclear protein and 50 μ g of BSA in each lane.

The (-) indicates that no extract was added to the probe.

ABA, the footprint on the ABREs became weaker. Thus, under these conditions, binding does not seem to be saturating and is therefore more like the experiment of Hill et al. (1996). Indeed, in excised embryos, the footprint on the ABREs was stronger in the wild type than it was in VP1-deficient embryos, suggesting that VP1 can have a positive effect on binding to the ABRE in vivo. One caveat of this conclusion is that excised *vp1* embryos have a germination capability different from that of the wild type (Robertson, 1955). The weaker footprints on the ABREs in excised VP1-deficient embryos could therefore reflect the altered germination of the mutant rather than a direct effect of VP1 deficiency.

Although we cannot exclude that different proteins bind to ABRE in the presence and in the absence of VP1, as proposed by Vasil et al. (1995), our observations are not in agreement with this possibility. The similarity of the footprints on *rab28* suggests that the same protein binds to the ABRE in the wild type and the *vp1* mutant. In addition, the same ABF was present in extracts from both wild-type and *vp1* embryos (P.K. Busk and M. Pagès, unpublished data). There could be a competition of different proteins with identical binding specificity on the ABRE. In this case, VP1 would specifically enhance binding of one of the two proteins to the ABRE. This is not in accordance with in vitro experiments in which VP1 enhances binding of all of the DNA binding proteins tested (Hill et al., 1996).

Developmental Activation of *rab28* through ABRE

VP1 is necessary but not sufficient for developmental expression of *rab28* because VP1 was detected in embryos as early as 10 DAP (McCarty et al., 1991), whereas *rab28* mRNA was not detected before 20 DAP (Pla et al., 1991; Niogret et al., 1996). ABA is also involved in induction of *rab28*, but a comparison of *rab28* expression in wild-type and ABA-deficient embryos showed that there is no clear correlation between the ABA level and the expression of *rab28* (Pla et al., 1991). The current data suggest that a developmental factor (the postabscission factor) is the primary inducer of genes expressed during late embryogenesis (Hughes and Galau, 1991; Giraudat et al., 1994).

In our study, we found an ABF in nuclear extracts whose activity correlates with the expression of *rab28*. The factor disappeared in excised embryos but could be maintained by ABA in the same way as the in vivo footprint on the ABREs of *rab28*. Therefore, it is a likely candidate for binding to the ABREs of *rab28*. This is supported by the fact that ABF bound more strongly to ABRE A than to ABRE B, which is in agreement with the relative strength of the in vivo footprints. These correlations suggest that the factor is relevant for the regulation of *rab28* in vivo, but additional work is required to clarify this point.

The factor is likely to belong to the bZIP family of transcription factors, because the pattern of protection and hy-

persensitivity on the two ABREs of the *rab28* promoter in vivo resembles the footprint of the bZIP proteins that bind to ACGT-containing *cis* elements (Lu et al., 1996).

The correlation of the *rab28* mRNA level with ABF activity but not with VP1 activity suggests that binding of ABF to the *rab28* promoter is a prerequisite for stimulation of transcription by VP1. That transcription factors can regulate the activity of VP1-like proteins has been demonstrated in bean (Chern et al., 1996). However, expression of *rab28* could also be regulated by a coactivator or a repressor that modifies the interaction of VP1 with the ABREs.

The disappearance of the ABF during germination suggests that it is embryo specific. It has been shown that ABRE A forms complexes of different mobility with nuclear proteins from embryos and leaves (Pla et al., 1993). The embryo-specific factor could be the maize homolog of the wheat protein EmBP-1, whose cDNA was cloned from an embryo cDNA library and binds to the ABRE (Guiltinan et al., 1990). The binding specificity of EmBP-1 (Niu and Guiltinan, 1994) is in agreement with a higher relative affinity for ABRE A than for ABRE B.

Regulation of *rab28* by ABA

Pla et al. (1993) showed that a fragment of 134 bp from the *rab28* promoter is sufficient for induction by ABA in rice protoplasts. Here, we have delineated four sequences in the same fragment that conferred ABA inducibility upon a reporter gene in transient transformation in maize embryonic callus. In vivo footprinting of maize embryos confirmed the importance of the four elements investigated. The expression study should be interpreted carefully because of the transient character of the transformation data. However, the results seem to be relevant for regulation of *rab28*, but it cannot be excluded that sequences outside of the examined region are also important.

During precocious germination of excised embryos, the footprints became weaker but did not disappear, even though *rab28* was not active (Niogret et al., 1996). In the *vp1* mutant in which only low levels of *rab28* mRNA are present (Pla et al., 1991), we observed the same footprints on the *rab28* promoter as were found in the wild type. In addition, the footprints in the *vp1* mutant did not change during induction with ABA. These results suggest that ABA and VP1 regulate *rab28* by modification of a preformed promoter complex. The induction of *rab28* by VP1 has been discussed above. ABA could stimulate *rab28* transcription via induced binding of a cofactor or by post-translational modifications, such as changes in protein phosphorylation. Both protein phosphatases (Leung et al., 1994; Meyer et al., 1994) and kinases (Knetsch et al., 1996) affect ABA-induced gene expression, indicating that ABA signal transduction involves phosphorylation and dephosphorylation events. Also, for the ABA-inducible gene *rab17*, there are footprints on the regulatory elements under uninduced conditions (Busk et al.,

1997). These results indicate that a modification of a pre-formed promoter complex is of major importance for ABA-induced transcription.

The two ABREs in the *rab28* promoter are necessary for both ABA- and VP1-mediated induction in embryonic callus. Although the data from transient transformation should be interpreted with care, the results are in accordance with the clear footprints on the two ABREs in embryos. In addition, in vivo footprinting of the *rab28* promoter in callus gave the same results (P.K. Busk and M. Pagès, unpublished data). In transient transformation, activation of the *rab28* promoter by VP1 was stronger in the presence than in the absence of ABA. This synergism between VP1 and ABA is typical of ABRE/G-box-mediated transcription (Hattori et al., 1992, 1995; Vasil et al., 1995) and mimicks the regulation in embryos. In embryos, the endogenous *rab28* gene is less inducible by ABA in the *vp1* mutant (Pla et al., 1991).

We are unable to explain the negative effect of VP1 on the activity of the ABRE A⁻ and ABRE B⁻ constructs. However, the effect was only seen without ABA. Under these conditions, the endogenous *rab28* gene is not active in callus (M.-F. Niogret and M. Pagès, unpublished data). The GRA element is an ABA- and water stress-inducible element from the *rab17* promoter (Busk et al., 1997). In this study, we found a sequence with 7 of 12 bp identical to GRA, which is involved in the ABA response and had a weak footprint in embryos.

The coupling element CE3 is present in the promoter of the barley gene *HVA1*, where it is necessary for induction by ABA (Shen et al., 1996). In the *rab28* promoter, the CE3-like element had a footprint in vivo, suggesting that it is involved in regulation of *rab28*. This is further supported by the importance of the element for induction by ABA in transient transformation. Shen et al. (1996) showed that CE3 together with an ABRE confer ABA inducibility upon a minimal promoter, whereas none of the elements functions alone. CE1 and an ABRE from *HVA22* also function together but cannot function alone (Shen and Ho, 1995). These results together with our finding that a combination of two ABREs in front of a 35S promoter was ABA inducible suggest that a combination of the ABRE with any other ABA-inducible element is sufficient to determine ABA response. Puente et al. (1996) described a similar regulation controlling light-responsive transcription.

METHODS

In Vivo Footprinting and DNA Purification

Wild-type embryos (*Zea mays*) of the pure inbred line W64A and homozygous *viviparous1* (*vp1*) mutants (Pla et al., 1991) were used for in vivo footprinting with dimethyl sulfate (DMS) within 15 min after excision from the cob or after 24 hr of incubation with 100 μ M abscisic acid (ABA) or with water (Busk et al., 1997). The DNA was purified and cleaved at DMS-modified guanines (Busk et al., 1997).

Ligation-Mediated Polymerase Chain Reaction

Ligation-mediated polymerase chain reaction (LMPCR) was performed as described previously (Busk et al., 1997). Two millimolar MgSO₄ was used in all PCR steps.

The sequence of oligonucleotides for LMPCR of the transcribed strand in the wild-type W64A is (in the order of first primer, second primer, and third primer) h1, 5'-CTACTACAGTACAGATCACGGCTTG-3'; h2, 5'-AGCTGCCTCTTGAGACTTGATGGTG-3'; and h3, 5'-GCCTCTTGAGACTTGATGGTGAGTGTGG-3'. Another primer set was used for the *vp1* mutant due to differences in the sequence of the promoter and is as follows: h3 and h4, 5'-GGATCGTTGCGATTATGCGGCGT-3'; and h5, 5'-TTGCGATTATATGCGGCGT(A/G)-CGGGAGAG-3'. For the nontranscribed strand, the oligonucleotides were i1, 5'-ACACGCATGCCCTGATGCTC-3'; i2, 5'-CGGGAGCTTCTTCATCCAGCTTG-3'; and i3, 5'-CGGGAGCTTCTTCATCCAGCTTG-3'.

Autoradiography and Scanning

AGFA Curix (AGFA, Leverkusen, Germany) or X-AR (Kodak, Rochester, NY) films were exposed at -70°C with an intensifying screen for 10 hr to 7 days.

All footprints were confirmed by scanning. For each experiment, several independent amplifications were performed by using LMPCR. No variation in band intensity was observed between amplifications. For quantitative evaluation, the films were scanned and the intensity of each peak was determined (Busk et al., 1997).

Plasmid Constructs

The wild-type promoter- β -glucuronidase (*GUS*) fusion construct was pMP130, which contains the *rab28* sequence from positions -393 to -60 fused to the cauliflower mosaic virus 35S -90 to +8 promoter in front of *GUS* and *nosT* (Pla et al., 1993).

Mutants were constructed by recombinant PCR as described previously (Cormack, 1992). The following mutants were obtained (sequences from 5' to 3', with wild-type bases in uppercase letters and mutated bases in lowercase letters and the numbering relative to the transcription start point): ABRE A⁻, (-152) CGCGCCTgcaGGGCATG (-135); ABRE B⁻, (-110) TCGTCTGaAttcCTCTC (-94); GRA⁻, (-142) TGGGCActCgagCGCGA (-126); and CE3⁻, (-128) CGACtCtagaCagCTTG (-112). The ABRE A⁻B⁻ mutant contained the ABRE A⁻ and ABRE B⁻ mutations.

The plasmid with ABRE A and ABRE B in front of a -90 35S promoter was constructed by annealing the oligonucleotides (sequences from 5' to 3', with *rab28* sequences in uppercase letters and other bases in lowercase letters) agcttGCCACGTGGGagatctgtcgacTCCACGTCTCc and tcgagGAGACGTGGAgtcgacagatcaCCC-ACGTGGCa. The double-stranded oligonucleotide was inserted in pMP130, which had been digested with HindIII and XhoI. The -90 35S promoter construct was made by cutting out the *rab28* promoter of pMP130 with Sall and XhoI and closing the vector fragment. All constructs were confirmed by DNA sequencing. 35S-Sh-Vp1 expresses VP1 under the control of a double 35S promoter and with an intron from the *shrunken* gene to increase expression. This plasmid is described by McCarty et al. (1991). The plasmid pRT-ex/s-int/s-LUC (Maas et al., 1991) was used as internal control in transformation assays.

Transient Transformation

Callus was made from the maize line Black Mexican Sweet and maintained as described by Vilardell et al. (1990) with 3 mg/mL 2,4-dichlorophenoxyacetic acid in the medium. Approximately 1 g of callus was spread on filter paper 1 week after replication and incubated overnight at 26°C in the dark. Four hours before bombardment, the callus was moved to medium with 200 mM mannitol. The callus was transformed by particle bombardment with 4 µg of the *rab28-GUS* plasmid and 2 µg of pRT-*ex/s-int/s-LUC* as an internal standard or with 3 µg of *rab28-GUS* plasmid DNA, 1.5 µg of 35S-Sh-Vp1, and 1.5 µg of pRT-*ex/s-int/s-LUC*, essentially as described by Klein et al. (1987).

Each sample was divided in two after transformation and incubated in medium with or without 100 µM ABA for 22 hr in the dark at 26°C before freezing in liquid nitrogen. Luciferase and GUS assays were done as previously described (Busk et al., 1997). Relative GUS activity is the reading of the GUS assay divided by the reading of the luciferase assay.

Nuclear Extracts

Nuclear extracts were prepared from embryos, as described by Busk and Pagès (1997).

Electrophoretic Mobility Shift Assays

The following DNA oligonucleotides were used for electrophoretic mobility shift assays (symbols are as given above): ABRE A, 5'-tgg-aagcttCGCGCCACGTGGGCATGagatct-3' and 5'-tggagatctCATGCC-CACGTGGCGCGaagctt-3'; ABRE A⁻, 5'-tggagcttCGCGCCtgcagg-GCATGagatct-3' and 5'-tggagatctCATGCCctgcaggCGCGaagctt3'; ABRE B, 5'-tggagcttGTCTCCACGTCTCTCGCagatct-3' and 5'-tgg-agatctGCGAGAGACGTGGAGACaagctt-3'; and ABRE B⁻, 5'-tggag-cttGTCTgaAttcCTCTCGCagatct-3' and 5'-tggagatctGCGAGAGga-aTtcAGACaagctt-3'.

The full-length oligonucleotides were purified by denaturing PAGE. Complementary oligonucleotides were annealed and purified in a nondenaturing polyacrylamide gel.

The double-stranded oligonucleotides were labeled with α-³²P-dATP (3000 Ci/mmol, Amersham) by filling in with the Klenow fragment of DNA polymerase I (Sambrook et al., 1989) and purified on a NAP5 column (Pharmacia), according to the manufacturer's instructions. Unlabeled competitor DNA was filled in with nonradioactive nucleotides.

The radioactive probe (10,000 cpm) was incubated with 5 to 45 µg of nuclear extract in 10 µL of 1 × binding buffer (25 mM Hepes, pH 7.8, 75 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 0.2 mM DTT, and 10% glycerol) and 150 ng of poly(dI-dC) for 20 min on ice before loading on a 1 × Tris-borate-EDTA, 5% (30:0.8 acrylamide-bis) polyacrylamide gel. Electrophoresis was at 10 V/cm at 4°C. In competition experiments, the nuclear extract was preincubated with the competitor in 8 µL of 1 × binding buffer and 120 ng of poly(dI-dC) for 10 min on ice before the radioactive probe was added.

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REFERENCES

- Busk, P.K., and Pagès, M. (1997). Microextraction of nuclear proteins for EMSA from single maize embryos. *Plant Mol. Biol. Rep.*, in press.
- Busk, P.K., Jensen, A.B., and Pagès, M. (1997). Regulatory elements in vivo in the promoter of the abscisic acid responsive gene *rab17* from maize. *Plant J.* **11**, 1285–1295.
- Chern, M.-S., Bobb, A.J., and Bustos, M.M. (1996). The regulator of *MAT2* (ROM2) protein binds to early maturation promoters and represses PvALF-activated transcription. *Plant Cell* **8**, 305–321.
- Cormack, B. (1992). Mutagenesis by the polymerase chain reaction. In *Current Protocols in Molecular Biology*, F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, eds (New York: Wiley Interscience), pp. 8.5.1–8.5.4 (suppl. 15).
- Giraudat, J., Parcy, F., Bertauche, N., Gosti, F., Leung, J., Morris, P.C., Bouvier-Durand, M., and Vartanian, N. (1994). Current advances in abscisic acid action and signalling. *Plant Mol. Biol.* **26**, 1557–1577.
- Giuliano, G., Pichersky, E., Malik, V.S., Timko, M.P., Scolnik, P., and Cashmore, A.R. (1988). An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. *Proc. Natl. Acad. Sci. USA* **85**, 7089–7093.
- Guilting, M.J., Marcotte, W.R., and Quatrano, R.S. (1990). A plant leucine zipper protein that recognizes an abscisic acid response element. *Science* **250**, 267–271.
- Hattori, T., Vasil, V., Rosenkrans, L., Hannah, L.C., McCarty, D.R., and Vasil, I.K. (1992). The *viviparous-1* gene and abscisic acid activate the *C1* regulatory gene for anthocyanin biosynthesis during seed maturation in maize. *Genes Dev.* **6**, 609–618.
- Hattori, T., Terada, T., and Hamasuna, S. (1995). Regulation of the *Osem* gene by abscisic acid and the transcriptional activator VP1: Analysis of *cis*-acting promoter elements required for regulation by abscisic acid and VP1. *Plant J.* **7**, 913–925.
- Hill, A., Nantel, A., Rock, C.D., and Quatrano, R.S. (1996). A conserved domain of the *viviparous-1* gene product enhances the DNA binding activity of the bZIP protein EmBP-1 and other transcription factors. *J. Biol. Chem.* **271**, 3366–3374.
- Hughes, D.W., and Galau, G.A. (1991). Developmental and environmental induction of *Lea* and *LeaA* mRNAs and the postabscission program during embryo culture. *Plant Cell* **3**, 605–618.
- Ingram, J., and Bartels, D. (1996). Molecular basis of dehydration tolerance in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 377–403.

- Izawa, T., Foster, R., and Chua, N.-H. (1993). Plant bZIP protein DNA binding specificity. *J. Mol. Biol.* **230**, 1131–1144.
- Klein, T.M., Wolf, E.D., Wu, R., and Sanford, J.C. (1987). High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature* **327**, 70–73.
- Knetsch, M.L.W., Wang, M., Snaar-Jagalska, B.E., and Heimovaara-Dijkstra, S. (1996). Absciscic acid induces mitogen-activated protein kinase activation in barley aleurone protoplasts. *Plant Cell* **8**, 1061–1067.
- Leung, J., Bouvier-Durand, M., Morris, P.C., Guerrier, D., Chedford, F., and Giraudat, J. (1994). *Arabidopsis* ABA response gene *ABI1*: Features of a calcium-modulated protein phosphatase. *Science* **264**, 1448–1452.
- Lu, G., Paul, A.-L., McCarty, D.R., and Ferl, R.J. (1996). Transcription factor veracity: Is GBF3 responsible for ABA-regulated expression of *Arabidopsis Adh*? *Plant Cell* **8**, 847–857.
- Maas, C., Laufs, J., Grant, S., Korfhage, C., and Werr, W. (1991). The combination of a novel stimulatory element in the first exon of the maize *Shrunken-1* gene with the following intron 1 enhances reporter gene expression up to 1000-fold. *Plant Mol. Biol.* **16**, 199–207.
- Marcotte, W.D., Jr., Russell, S.H., and Quatrano, R.S. (1989). Absciscic acid-responsive sequences from the *Em* gene of wheat. *Plant Cell* **1**, 969–976.
- McCarty, D.R. (1995). Genetic control and integration of maturation and germination pathways in seed development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 71–93.
- McCarty, D.R., Hattori, T., Carson, C.B., Vasil, V., Lazar, M., and Vasil, I.K. (1991). The *Viviparous-1* developmental gene of maize encodes a novel transcriptional activator. *Cell* **66**, 895–905.
- McKendree, W.L., Jr., and Ferl, R.J. (1992). Functional elements of the *Arabidopsis Adh* promoter include the G-box. *Plant Mol. Biol.* **19**, 859–862.
- Meyer, K., Leube, M.P., and Grill, E. (1994). A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. *Science* **264**, 1452–1455.
- Mundy, J., Yamaguchi-Shinozaki, K., and Chua, N.-H. (1990). Nuclear proteins bind conserved elements in the absciscic acid-responsive promoter of a rice *RAB* gene. *Proc. Natl. Acad. Sci. USA* **87**, 1406–1410.
- Niogret, M.F., Culiáñez-Macià, F.A., Goday, A., Albà, M.M., and Pagès, M. (1996). Expression and cellular localization of *rab28* mRNA and Rab28 protein during maize embryogenesis. *Plant J.* **9**, 549–557.
- Niu, X., and Guitinan, M.J. (1994). DNA binding specificity of the wheat bZIP protein EmBP-1. *Nucleic Acids Res.* **22**, 4969–4978.
- Niu, X., Adams, C.C., Workman, J.L., and Guitinan, M.J. (1996). Binding of the wheat basic leucine zipper protein EmBP-1 to nucleosomal binding sites is modulated by nucleosome positioning. *Plant Cell* **8**, 1569–1587.
- Parcy, F., Valon, C., Raynal, M., Gaubier-Comella, P., Delseny, M., and Giraudat, J. (1994). Regulation of gene expression programs during *Arabidopsis* seed development: Roles of the *ABI3* locus and of endogenous absciscic acid. *Plant Cell* **6**, 1567–1582.
- Pla, M., Gómez, J., Goday, A., and Pagès, M. (1991). Regulation of the absciscic acid-responsive gene *rab28* in maize *viviparous* mutants. *Mol. Gen. Genet.* **230**, 394–400.
- Pla, M., Vilardell, J., Guitinan, M.J., Marcotte, W.R., Niogret, M.F., Quatrano, R.S., and Pagès, M. (1993). The *cis*-regulatory element CCACGTGG is involved in ABA and water-stress responses of the maize gene *rab28*. *Plant Mol. Biol.* **21**, 259–266.
- Puente, P., Wei, N., and Deng, X.W. (1996). Combinatorial interplay of promoter elements constitutes the minimal determinants for light and developmental control of gene expression in *Arabidopsis*. *EMBO J.* **15**, 3732–3743.
- Rigaud, G., Roux, J., and Pictet, R. (1991). In vivo footprinting of rat TAT gene: Dynamic interplay between the glucocorticoid receptor and a liver-specific factor. *Cell* **67**, 977–986.
- Robertson, D.S. (1955). The genetics of vivipary in maize. *Genetics* **40**, 745–760.
- Salinas, J., Oeda, K., and Chua, N.-H. (1992). Two G-box-related sequences confer different expression patterns in transgenic tobacco. *Plant Cell* **4**, 1485–1493.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
- Schultz, T.F., Spiker, S., and Quatrano, R.S. (1996). Histone H1 enhances the DNA binding activity of the transcription factor EmBP-1. *J. Biol. Chem.* **271**, 25742–25745.
- Shen, Q., and Ho, T.-H.D. (1995). Functional dissection of an absciscic acid (ABA)-inducible gene reveals two independent ABA-responsive complexes each containing a G-box and a novel *cis*-acting element. *Plant Cell* **7**, 295–307.
- Shen, Q., Zhang, P., and Ho, T.-H.D. (1996). Modular nature of absciscic acid (ABA) response complexes: Composite promoter units that are necessary and sufficient for ABA induction of gene expression in barley. *Plant Cell* **8**, 1107–1119.
- Suzuki, M., Kao, C.Y., and McCarty, D.R. (1997). The conserved B3 domain of VIVIPAROUS1 has a cooperative DNA binding activity. *Plant Cell* **9**, 799–807.
- Vasil, V., Marcotte, W.R., Jr., Rosenkrans, L., Cocciolone, S.M., Vasil, I.K., Quatrano, R.S., and McCarty, D.R. (1995). Overlap of Viviparous1 (VP1) and absciscic acid response elements in the *Em* promoter: G-box elements are sufficient but not necessary for VP1 transactivation. *Plant Cell* **7**, 1511–1518.
- Vilardell, J., Goday, A., Freire, M.A., Torrent, M., Martínez, M.C., Torné, J.M., and Pagès, M. (1990). Gene sequence, developmental expression, and protein phosphorylation of RAB-17 in maize. *Plant Mol. Biol.* **14**, 423–432.
- Weisshaar, B., Armstrong, G.A., Block, A., Da Costa e Silva, O., and Hahlbrock, K. (1991). Light-inducible and constitutively expressed DNA-binding proteins recognizing a plant promoter element with functional relevance in light responsiveness. *EMBO J.* **10**, 1777–1786.
- Williamson, J.D., and Scandalios, J.G. (1992). Differential response of maize catalases to absciscic acid: Vp1 transcriptional activator is not required for absciscic acid-regulated *Cat1* expression. *Proc. Natl. Acad. Sci. USA* **89**, 8842–8846.