

## $\beta$ -Phaseolin gene activation is a two-step process: PvALF-facilitated chromatin modification followed by abscisic acid-mediated gene activation

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**ABSTRACT** We have shown previously that a rotationally and translationally positioned nucleosome is responsible for the absence of transcriptional expression from the phaseolin (*phas*) gene promoter in leaf tissue and that the repressive chromatin structure is disrupted on transcriptional activation during embryogenesis. To investigate how the chromatin structure is modified, we ectopically expressed PvALF, a putative seed-specific *phas* activator, in leaf tissue of a tobacco line transgenic for a chimeric *phas/uidA* construct. DNase I footprinting *in vivo* revealed that the ectopic expression of PvALF resulted in remodeling of the chromatin architecture over the TATA region of the *phas* promoter but did not lead to transcriptional activation in the absence of abscisic acid (ABA). Treatment of the transgenic tobacco leaves with ABA in the absence of PvALF neither alleviated the repressive chromatin architecture nor activated transcription. However, in the presence of PvALF, high levels of  $\beta$ -glucuronidase expression were obtained on exposure of leaves to ABA. These results reveal that expression from the *phas* promoter involves at least two discrete steps: chromatin potentiation by PvALF followed by ABA-mediated transcriptional activation.

Phaseolin, the major seed storage protein of bean (*Phaseolus vulgaris*), is encoded as a small multigene family (1). Extensive studies of one member of this family,  $\beta$ -phaseolin (*phas*), have established that it is highly expressed during embryogenesis and that chromatin architecture participates in its total transcriptional silence in vegetative tissues (2, 3). Phaseolin synthesis has been shown to be stimulated by exogenous abscisic acid (ABA) in intact embryos (4), and Bustos *et al.* (5) recently showed that exogenous ABA treatment stimulates *phas* gene expression in intact embryos of transgenic tobacco. These observations, together with the findings that the *Glb12S* storage protein gene of tobacco is similarly regulated during seed development (5) and that the concentration of free ABA in the embryo rises at the time of rapid phaseolin synthesis in bean (6), suggest that, as has been found for several other seed protein genes, ABA plays an important role in transcriptional activation of *phas*. Indeed, ABA is known to be involved in diverse physiological processes that include seed maturation and germination and the adaptation of vegetative tissues to environmental stresses (7–9).

Intensive studies have been conducted during the past decade to unravel the mechanism by which ABA participates in physiological processes (7, 10, 11), and it is now becoming clear that there are at least two distinct pathways for ABA-inducible expression. One is tissue-nonspecific, and genes regulated through this pathway include those involved in stress-related processes and some late embryo-absent genes

that are active during seed maturation, e.g., *rab16A* (12) and *rd29* (13). The second pathway is tissue-specific, and gene expression can be induced by ABA only in those tissues where a specific activator is available. Genes regulated through this pathway include seed storage protein genes, e.g., *HaG3-A* (14) and some late embryo-absent genes that are activated and expressed during embryogenesis.

In maize, it has been postulated that ABA-responsive transcription of the *C1* anthocyanin regulatory gene is specified by VP1 (15), the product of the *Viviparous-1* (*Vp1*) locus, the expression of which is restricted to seed tissues (16). This hypothesis is supported by the observation that ectopic expression of *ABI3* (which encodes an *Arabidopsis* homolog of VP1) leads to the accumulation of mRNAs for some seed-specific genes in response to exogenous ABA in vegetative tissues of transgenic *Arabidopsis* plants. Specifically, Parcy *et al.* (17) have shown that the transcription of mRNAs for the storage protein At2S and the late embryogenesis-abundant AtEm 1 is ABA-inducible in vegetative tissues of transgenic plants carrying transcriptional fusions between the double-enhanced *CaMV 35S* promoter and *ABI3* cDNA, but not in wild-type vegetative tissues.

Although we have previously shown that exogenous ABA treatment does not activate transcription from *phas* constructs in calli generated from vegetative tissues of transgenic tobacco plants (2), the findings discussed above stimulated us to appraise the possibility that application of ABA in the presence of a transcriptional activator might relieve the chromatin constraints known to participate in the spatial regulation of *phas* gene expression (3). We conjectured that such relief might permit expression from the *phas* promoter in vegetative tissues analogous to the transient expression obtained on bombardment of *phas/uidA* constructs (methylated or non-methylated) into bean or tobacco leaves (2). Although Bobb *et al.* (18) have shown enhanced trans-activation of the *phas* promoter in similar experiments by transient ectopic expression of *PvAlf*, stable expression from chromosomal copies of the *phas* promoter has not been attained previously. PvALF is a *P. vulgaris* *ABI3*-like factor that, like its counterpart in *Arabidopsis*, is encoded by a single-copy gene, and its expression is seed-specific and developmentally regulated during embryogenesis (18). PvALF therefore appeared to be an excellent candidate for a factor that, in the presence of ABA, might be capable of transcriptional activation of *phas* in vegetative tissues.

To test the ability of PvALF to activate expression from *phas* in its chromatin environment in vegetative tissue, we double-

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: GUS,  $\beta$ -glucuronidase; ABA, abscisic acid; PR, progesterone receptor; PvALF, *Phaseolus vulgaris* ABI3-like factor; LMPCR, ligation-mediated PCR; *phas*, phaseolin gene; *uidA*, the gene encoding GUS.

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transformed plant 58.1A [containing a *-1470phas/uidA* gene; van der Geest *et al.* (19)] with a *35S/PvAlf* construct. Our results show that, when PvALF is expressed in vegetative tissue, the *phas* promoter is converted from a tissue-specific into a tissue nonspecific ABA-inducible promoter. We also show that this conversion is related to a change in the chromatin configuration of the *phas* promoter.

## MATERIALS AND METHODS

**Cloning and Plant Transformation.** A 2.3 kb *Sali*-*Sma*I fragment of pJIT-PvAlf (18), kindly provided by Mauricio Bustos (Univ. of Maryland, Baltimore County), that contains the *PvAlf*-coding region was cloned into the shuttle vector pART7 (20). A 4.7-kb *Not*I fragment of the resulting plasmid (pART7-PvAlf), which contained the chimeric gene construct *35S/PvAlf/inos*, was subsequently cloned into pBJ49 (B. Jansen, unpublished data) to yield pBJ49-PvAlf that was transformed into *Agrobacterium tumefaciens* strain LBA4404 via electroporation.

A leaf disc from tobacco line 58.1A, homozygous for *-1470phas/uidA* (19), was retransformed by inoculation for 10 min with LBA4404:BJ49-PvAlf grown to saturation in LB medium. The inoculated leaf discs were then incubated for 2 days on Murashige-Skoog (MS) medium (21) at 25°C under 16 h light/8 h dark. Subsequently, the discs were transferred to a shoot-inducing medium (MS + 2 µg/ml 6-benzyladenine + 0.01 µg/ml naphthaleneacetic acid) containing 25 mg/liter hygromycin and 150 mg/liter Claforan (equivalent to cefotaxime, Hoechst-Roussel). Regenerated young shoots were transferred to rooting medium (half-strength MS medium containing 25 mg/liter hygromycin and 150 mg/liter Claforan). Rooted plants were transferred to soil and grown to maturity. Genomic DNA from transformants (lines PvAlf-1 to -14) was analyzed for insertion of the intact transgene by using PCR or genomic DNA blot analysis. Line PvAlf-14 contained a single intact copy of the transgene, was phenotypically normal, and was chosen for use in most of the studies reported here.

**ABA Induction.** Seeds collected from either 58.1A plants or from line PvAlf-14 were surface-sterilized with 20% (vol/vol) household bleach and then rinsed three times in sterile water. Seedlings (10 day) or leaves from seedlings (30 day), selected on MS medium containing either 400 µg/ml kanamycin or 50 µg/ml hygromycin, were incubated in liquid basal MS medium with or without 200 µM ABA ( $\pm$  cis/trans isomer; Sigma) for 2 days (12 h for the RNase-protection experiment) in darkness with gentle shaking at room temperature.

**Fluorometric and Histochemical  $\beta$ -glucuronidase (GUS) Assays.** GUS activity was determined according to Jefferson *et al.* (22). For the fluorometric assay, leaves or callus were homogenized in GUS extraction buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0/10 mM EDTA/0.1% sarkosyl, 0.1% Triton X-100/10 mM 2-mercaptoethanol) and centrifuged for 5 min in a microcentrifuge. A 200-µl volume of extract was then mixed with 200 µl of substrate solution (GUS extraction buffer + 4-methylumbelliferyl  $\beta$ -D-glucuronide: 4-MUG, Fluka) and incubated at 37°C; 100-µl aliquots were removed at 0, 60, or 120 min, and the reaction was terminated by addition of 900 µl of Na<sub>2</sub>NO<sub>3</sub>. Fluorescence was measured on a fluorometer (model TKO 100, Hoefer). Protein concentrations were determined by using the colorimetric assay of Bradford (23). Specific GUS activity was calculated as pmol of 4-MU h<sup>-1</sup>·µg<sup>-1</sup> protein. For histochemical GUS analysis, seedlings or leaves were immersed in GUS staining solution [50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0/1 mM EDTA/3 mM K ferricyanide/3 mM K ferrocyanide/2 mM X-Gluc (5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide)] and vacuum-infiltrated to facilitate penetration. Chlorophyll was removed from stained leaf tissues by immersion in 95% ethanol solution at room temperature for 12–48 hr.

**RNase Protection Assay.** Several antisense constructs for generating riboprobes were prepared by subcloning a fragment containing the 3' end of the *uidA*, *PvAlf*, or *hpt* coding region into pPCR-Script (Stratagene). Antisense *uidA*, *PvAlf*, *hpt*, and *18S* riboprobes of 310, 430, 345, or 200 nt, respectively, were synthesized by transcription *in vitro*, using T3 or T7 RNA polymerase on a *Hind*III- or *Eco*R1-linearized plasmid.

RNase protection assays were performed in reactions containing 10 µg of total RNA by using an RPAII kit (Ambion, Austin, TX). RNA from 58.1A was used as a negative control for *uidA* and *PvAlf* mRNAs. The protected fragments were analyzed by electrophoresis on a 5% polyacrylamide-8 M urea gel, by using <sup>32</sup>P-labeled *in vitro*-transcribed RNA century marker (Ambion, Austin, TX) as a size standard.

**Isolation of Nuclei.** Nuclei were isolated essentially as described (3). Briefly, leaves were powdered in a mortar and pestle by using liquid nitrogen, treated with nuclei isolation buffer NIB1 (3) and the extract that filtered through a 20-µm mesh sieve was loaded onto a 20–80% Percoll (Sigma) step gradient. Nuclei were removed from the 20–80% Percoll interface and washed in NIB2 (3).

**Ligation-Mediated PCR.** For first-strand synthesis, 2 µg of DNase I-digested DNA and 0.5 pmol of primer 1 (5'-CTCT-TCGCCACCTCAATTC-3') were mixed with 3 µl of 10× Vent polymerase buffer (New England BioLabs) and 2 mM of each dNTP in a final volume of 30 µl. After heating to 95°C for 5 min, the primer was annealed (30 min at 60°C) and extended (10 min at 76°C). For the ligation of linker, prepared by using the two oligonucleotides and hybridization conditions described by Mueller and Wold (24), the sample was transferred to ice and 20 µl of buffer (110 mM Tris, pH 7.5/18 mM MgCl<sub>2</sub>/50 mM DTT/0.0125% BSA) and 25 µl of ligation mix (10 mM MgCl<sub>2</sub>/20 mM DTT/3 mM ATP/0.0025% BSA/100 pmol of linker/3 units of T4 DNA ligase) were added sequentially. After ligation of the linker and ethanol precipitation, DNA was dissolved in 85 µl of H<sub>2</sub>O. Subsequently, 10 µl of 10× PCR buffer (10 mM Tris-HCl, pH 8.0/50 mM KCl/1.5 mM MgCl<sub>2</sub>/0.01% gelatin/2 mM each dNTP/0.1% Triton X-100), 20 pmol of gene specific primer P2 (5'-CTTCACTCAAC-ACACGTCACCTGC-3'), 20 pmol of linker primer, and 2.5 units of *Taq* DNA polymerase were added. Samples were amplified by 20 cycles of PCR, and primer extension was done by using *Taq* DNA polymerase and [ $\gamma$ -<sup>32</sup>P]-radiolabeled gene-specific primer 3 (5'-CAACACACGTCACCTGCATATG-CGTGTC-3'). After precipitation, the DNA was dissolved in 20 µl of loading buffer, and nick sites were visualized by denaturing gel electrophoresis of 2- to 5-µl samples and analyzed on a Fujix BAS 2000 Bio-Imaging Analyzer.

## RESULTS

**Ectopic Expression of PvALF Makes the *phas* Promoter ABA-Inducible in Vegetative Tissues.** In *Arabidopsis*, it has been shown that ectopic expression of *ABI3* can induce the expression of some seed protein genes in vegetative tissues (17). To investigate whether PvALF, an *ABI3*-like protein from bean, can activate transcription from the *phas* promoter in leaf and other tissues of tobacco, we examined the expression of GUS in 14 independent lines of tobacco doubly transformed with *35S/PvAlf* and *-1470phas/uidA* (PvAlf-1 to -14, see *Materials and Methods*). Both fluorimetric (4-methylumbelliferyl- $\beta$ -D-glucuronide; MUG) and histochemical (GUS) assays of leaf tissue from these plants failed to detect the presence of GUS. However, in response to ABA treatment, GUS activity was detected in leaves from 11 of the 14 lines. As shown in Figs. 1 and 2 A–D, both MUG assays and GUS staining showed substantial amounts of GUS accumulation in ABA-treated leaves of line PvAlf-14. This line contained a single, intact, copy of the *35S/PvAlf* transgene and was chosen for all further studies. The level of GUS activity was found to



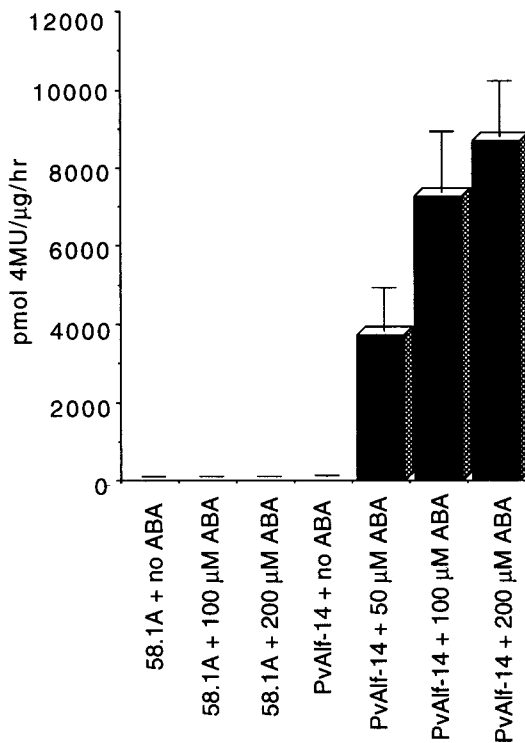


FIG. 1. Activation of the *phas* promoter requires both PvALF and ABA. Expression is shown as MUG activity in leaves of isogenic lines 58.1A (-1470*phas/uidA*) and PvAlf-14 (-1470*phas/uidA*; *CaMV35S/PvAlf*). Leaves were cultured in MS solution containing the indicated concentrations of ABA. The results are the average of at least five replicates.

be positively correlated with the concentration of ABA used (Fig. 1).

Although *phas* expression in the line PvAlf-14 was highly responsive to ABA treatment, ABA cannot by itself activate *phas* expression in the absence of PvALF. This is evident from the data shown in Fig. 1 and Fig. 2 *A* and *B*, where the *phas* promoter is inactive in leaf tissue of plant 58.1 (in which *PvAlf* is not ectopically expressed), whether treated with ABA or not. Similarly, expression of *PvAlf* does not lead to transcriptional activation of *phas* in the absence of ABA (Fig. 3C). In contrast, in the presence of both PvALF and ABA, strong expression of GUS was obtained in vegetative tissues (leaf, stem, root) of PvAlf-14 (Fig. 1 and Fig. 2D). The requirement for the combined presence of PvALF and ABA for transcriptional activation of the *phas* promoter is further illustrated by the RNase protection assays shown in Fig. 3. A strong signal, representing protection of *uidA* mRNA, was evident for tissues in which both PvALF and ABA were present, but no transcript was detected by this sensitive assay when either of these factors were absent (Fig. 3, compare lane 4 and lanes 1–3).

The lack of expression from the *phas* promoter in non-ABA-treated vegetative tissues should not be due to the absence of PvALF because *PvAlf* was under the control of the *CaMV35S* promoter. Indeed, when RNase protection assays were conducted for *PvAlf* transcripts, a similar amount of *PvAlf* mRNA was detected for both ABA-treated and -nontreated plants (Fig. 3, lanes 3 and 4). The low levels of signal apparent in the 58.1A plants in Fig. 3, lanes 1 and 2, probably reflect the fact that the riboprobe contained sequences that are homologous to those of factors present in leaf tissues. For example, RAP 2.8 has sequence similarity with PvALF and is present in both reproductive and vegetative tissues (25).

Exposure of PvAlf-14 tissues to auxin, cytokinin, gibberellic acid, ethylene, or methyl jasmonic acid did not induce *phas*

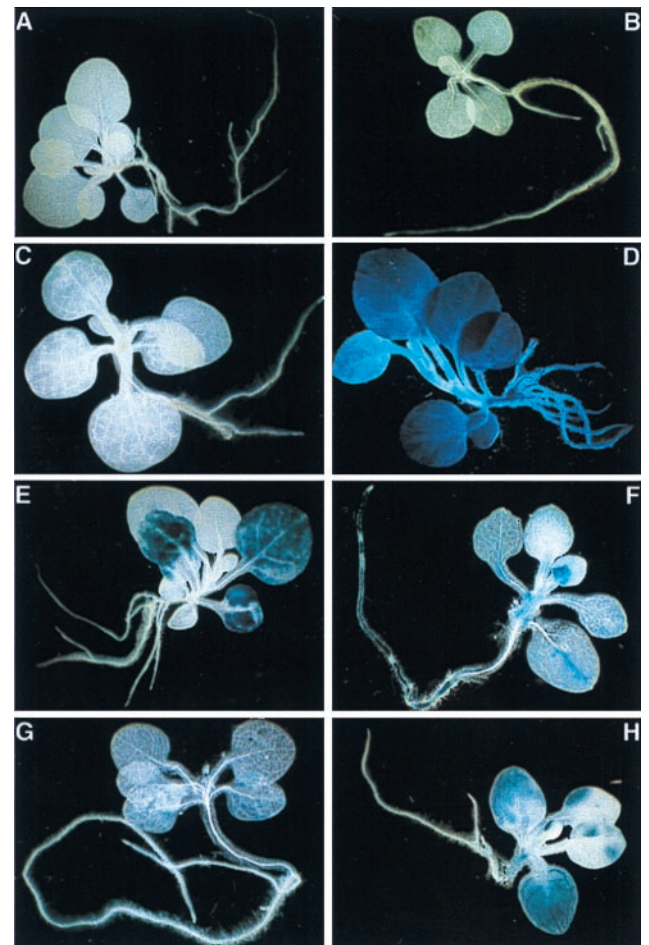


FIG. 2. Histochemical staining patterns for transgenic tobacco lines containing various *uidA* constructs with or without ABA treatment. For ABA treatment, the leaves were cultured in liquid MS medium containing 200 μM ABA. Control leaves were cultured in liquid MS medium alone. Blue color reveals accumulated GUS protein. (A) 58.1A (-1470*phas/uidA*). (B) 58.1A treated with ABA. (C) PvAlf-14 (-1470*phas/uidA*; *CaMV35S/PvAlf*). (D–G) PvAlf-14 treated with ABA. (H) Plant transgenic for *CaMV35S/uidA*.

expression (Fig. 4). These experiments confirm our previous results (2) and also suggest that the role ABA plays in *phas* gene regulation cannot be replaced by other plant growth regulators.

Although histochemical detection of GUS was readily obtained in leaves of PvAlf-14 exposed to ABA, the pattern of expression was far from uniform. By using similar conditions of ABA treatment and histochemical staining for GUS, seedlings were obtained with uniformly and darkly blue-stained leaves and roots (Fig. 2D), some with leaves that were mostly stained and other leaves that were completely devoid of GUS expression (Fig. 2E). Some seedlings had very limited regions of staining in the leaf but strong staining in the roots (Fig. 2F), and others had very limited regions of staining in the leaves and roots (Fig. 2G). These differences persisted in many (>20) experiments despite taking great care to ensure that the vacuum applied and time of infiltration were similar for each sample within an experiment and that each sample was similarly exposed to the ABA-containing medium. Although it is possible that the irregular staining patterns observed in ABA-treated PvAlf-14 plants (Fig. 2) may result from aberrant silencing of the *PvAlf* transgene, a more likely reason is that, as has been demonstrated previously (26, 27), the *CaMV 35S* promoter is not constitutively expressed at all times in all tissues and, hence, PvALF was not uniformly expressed in leaf

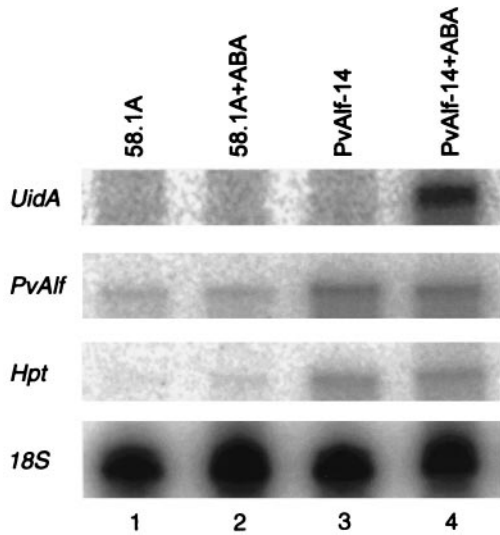


FIG. 3. RNA expression in the presence or absence of PvALF and ABA. RNase protection assays are shown for *uidA*, *PvAlf*, *hpt*, and *18S* in ABA-treated or nontreated leaves of transgenic tobacco lines 58.1A (-1470*phas/uidA*) and PvAlf-14 (-1470*phas/uidA*; *CaMV35S/PvAlf*).

tissues of PvAlf-14. Indeed, GUS staining of tobacco transgenic for *35S/uidA* was found to give an uneven pattern (Fig. 2H) similar to that obtained for ABA-treated PvAlf-14 plants (Fig. 2E).

**Evidence That PvALF Is Involved in Chromatin Remodeling of the *phas* Promoter.** Previously, we showed that a nucleosome is rotationally positioned on the *phas* promoter in leaf tissues (3). Although it is clear that the nucleosome is disrupted on *phas* promoter activation during embryogenesis, the key question remains as to what brings about this disruption. Our results infer that modification of chromatin structure is triggered by PvALF, by ABA, or by a combination of PvALF and ABA. To find out which of these alternatives starts the process of nucleosome disruption before transcriptional activation,

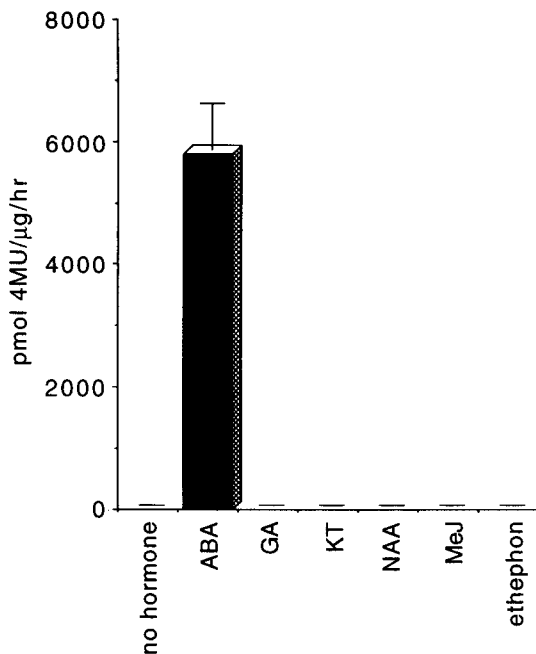


FIG. 4. MUG activity of PvAlf-14 under different hormone regimes. ABA, abscisic acid 200 μM; GA, gibberellic acid 10 μM; KT, kinetin 50 μM; NAA, naphthaleneacetic acid 50 μM; MeJ, methyl jasmonate 200 μM; ethephon 1 mM. The results are the average of five replicates.

DNase I mapping *in vivo* was undertaken for the top strand of the TATA region of the *phas* promoter in leaf tissue for both 58.1A and PvAlf-14 plants cultured in the presence or absence of ABA. As reported previously (3), a 10-bp repeat pattern of cleavage sites characteristic of rotational nucleosome positioning was observed for 58.1A leaf tissue (Fig. 5, lane 2). An almost identical footprinting profile was obtained for 58.1A leaves treated with ABA (Fig. 5, lane 3), suggesting that ABA alone cannot initiate chromatin remodeling. A different chromatin profile was observed for PvAlf-14 in the absence of ABA (Fig. 5, lane 4). In addition to the differences in the position of hyperreactive residues, cutting at hyperreactive sites in the *phas* initiator region is stronger and the accessibility of adjacent nucleotides for cleavage appears to be much greater in leaf nuclei from PvAlf-14 than that from 58.1A. Because 58.1A and PvAlf-14 are isogenic lines, their differing DNase I *in vivo* footprint profiles suggest that PvALF can initiate the chromatin remodeling process. As expected, the footprint profile for the active *phas* promoter in PvAlf-14 leaves treated with ABA is similar to that for the active promoter in seed tissues (3). However, protection over the TATA boxes (Fig. 5, lane 5) was not as obvious in leaf as it is in seed, probably reflecting

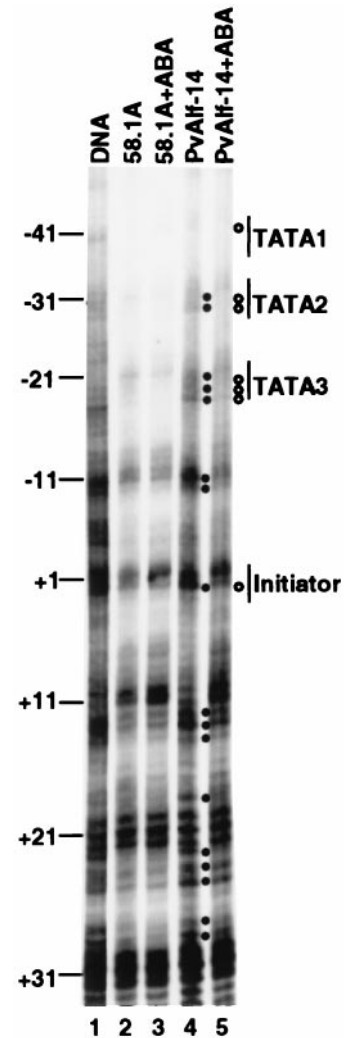


FIG. 5. Increased nuclease accessibility to the *phas* promoter in the presence of PvALF. DNase I footprinting *in vivo* of the top strand of the *phas* promoter proximal region. Nuclei were isolated from leaves of 58.1A and PvAlf-14 plants incubated in the presence or absence of ABA. DNA was extracted and subjected to ligation-mediated PCR. ● indicates hyperreactive residues, relative to the basal state (58.1A), in PvAlf-14 in the absence of ABA; ○ denotes protected regions of the *phas* promoter in PvAlf-14 in the presence of ABA.

the fact that *phas* activation was not uniform in the PvAlf-14 leaf tissues analyzed (see Fig. 2).

## DISCUSSION

Although the *phas* promoter is not inducible by ABA or by several other hormonal and environmental regimes in vegetative tissues (2), it has been shown previously that exogenous ABA stimulates *phas* gene expression in intact embryos (4, 5). Our data indicate that this stimulation results from the presence of PvALF in such tissues and show that ectopically expressed PvALF can make the *phas* promoter ABA-inducible in vegetative tissues, where PvALF is usually absent. Importantly, by using DNase I footprinting *in vivo*, we show that PvALF can modify the *phas* promoter (Fig. 5), making it much more accessible to DNase I cleavage. Although this chromatin modification does not by itself activate the *phas* promoter, it appears to potentiate it, thereby permitting the binding of an ABA-activated transcription factor (or factors) to its recognition site. Thus, the onset of transcription on the *phas* promoter can be separated into two discrete steps: potentiation, in which PvALF mediates a change in chromatin architecture; and activation, in which an ABA-modified factor (or factors) binds the remodeled chromatin and recruits the transcription machinery.

Similar observations to those obtained for PvALF have been described for GAGA factor, which is able to establish the chromatin structure of the *hsp70* promoter in conjunction with the nucleosome remodeling complex NURF so that it becomes heat-inducible and can be activated on binding of HSF (28). Progesterone receptor (PR) is another example of a factor that can remodel chromatin structure without activating gene expression. In the gr-/PR+ cell line where PR is constitutively present, Nuc-B of the mouse mammary tumor virus promoter is constitutively organized into a more open conformation. This potentiated mouse mammary tumor virus promoter exhibits only basal transcription activity but becomes fully active in the presence of hormone (29). Although PvALF may assume the function of GAGA factor and PR, we have not excluded the possibility that a factor (or factors) induced (or recruited) by PvALF is the key trigger for chromatin remodeling. It is also possible that the function of PvALF is to enhance the DNA binding activity of other transcription factors, as in the case of enhancement of the binding of bZIP protein EmBP-1 to *Em* promoter by VP1 (30).

A gene can exist in a variety of different chromatin states, depending on the stage of the cell cycle and the properties of the proteins with which it interacts. Some factors, such as HP1, contribute to the formation of inactive chromatin (31), whereas others, like PvALF, potentiate the promoter for activation. Potentiation of the *phas* promoter can be achieved in two ways: one is through replication-coupled chromatin structure changes in which transcription factor binding outcompetes chromatin assembly; the other is chromatin remodeling independent of DNA replication (32). Whereas PvALF may modify chromatin structure by recruiting a nucleosome remodeling complex such as SWI/SNF (33) or HAT (34), in the case of the plants transgenic for *35S/PvAlf*, it could alternatively take advantage of the many windows of opportunity afforded by the numerous replication cycles during germination and seedling growth because PvALF should be constitutively available. The concept that potentiation of the *phas* promoter involves the replication process is attractive because DNA replication and endoreduplication are characteristic for embryogenesis in bean (35), where the native *phas* gene is active. Indeed, detailed studies of the homologous vicilin storage protein of pea clearly established that all cells seen to contain vicilin have an elevated DNA level and that the DNA replication inhibitor aphidicolin affected vicilin deposition (36). Whereas vicilin-containing cells were distributed throughout the entire embryo in embryos grown *in vitro* in

aphidicolin-free media, no vicilin-containing cells were observed in the axis region of sectioned aphidicolin-treated embryos (36, 37). In preliminary experiments, we bombarded mature tobacco leaves of 58.1A with *35S/PvAlf* to see whether transiently expressed PvALF could activate the *phas* promoter in the presence of ABA. Because there is no DNA replication in mature tobacco leaf cells, any potentiation of the *phas* promoter by PvALF would be attributed to remodeling rather than to replication. In contrast to our findings with stably integrated *35S/PvAlf*, no expression was seen in these experiments, which provide support for the association of DNA replication and initiation of expression from seed protein promoters. Further experimentation is clearly needed to explore this possibility.

Our findings provide insight to mechanisms that may be widely used by tissue-specific promoters containing elements (such as RY repeats) that interact with VP1-like factors. Whereas the *phas* promoter probably represents an extreme case, it now seems likely that many of these promoters are constrained by a chromatin architecture that requires restructuring prior to transcriptional activation. It is of special interest that the potentiation step, exemplified by PvALF-mediated modification of the *phas* promoter, can be entirely separated from transcriptional activation, mediated by ABA in the case of *phas*. This insight helps to explain the many variations in spatial and temporal regulation encountered for promoters containing RY repeats. For example, differences in sensitivity of factors in the ABA-stimulated cascade to hormone levels and the generation of ABA in specific plants and tissues can result in dramatically different phenotypic expression patterns. It will be important to learn the mechanism by which PvALF restructures chromatin and to define the steps and factors involved in ABA response.

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