Ordered Histone Modifications Are Associated with Transcriptional Poising and Activation of the phaseolin Promoter

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The phaseolin (phas) promoter drives copious production of transcripts encoding the protein phaseolin during seed embryogenesis but is silent in vegetative tissues, in which a nucleosome is positioned over its three-phased TATA boxes. Transition from the inactive state in transgenic Arabidopsis thaliana leaves was accomplished by ectopic expression of the transcription factor Phaseolus vulgaris ABI3-like factor (ALF) and application of abscisic acid (ABA). Placement of hemagglutinin-tagged ALF expression under the control of an estradiol-inducible promoter permitted chromatin immuno-precipitation analysis of chronological changes in histone modifications, notably increased acetylation of H3-K9 and H4-K12, as phas chromatin was remodeled (potentiated). A different array of changes, including acetylation of H3-K14 and methylation of H3-K4, was found to be associated with ABA-mediated activation. Thus, temporal separation of phas potentiation from activation revealed that histone H3 and H4 Lys residues are not globally hyperacetylated during phas expression. Whereas decreases in histone H3 and H4 levels were detected during ALF-mediated remodeling, slight increases occurred after ABA-mediated activation, suggesting the restoration of histone–phas interactions or the replacement of histones in the phas chromatin. The observed histone modifications provide insight into factors involved in the euchromatinization and activation of a plant gene and expand the evidence for histone code conservation among eukaryotes.

INTRODUCTION

Although a wealth of information exists concerning the overall regulation of transcription from eukaryotic promoters, much remains to be learned regarding how specific promoters are selected for activation. In plants, the phaseolin (phas) promoter provides an excellent system to explore this challenge, because it is silent in all vegetative tissues of the bean (Phaseolus vulgaris) plant (van der Geest et al., 1995) but becomes exceptionally transcriptionally active during the development of the seed embryo (Hall et al., 1999; Li et al., 2001).

The contrast between the complete failure of phas-gus constructs to express β-glucuronidase (GUS) in vegetative tissues when stably integrated into the genome and the abundant expression of GUS from the same constructs when transiently inserted into leaves as naked DNA (Frisch et al., 1995) provided compelling circumstantial evidence for the involvement of chromatin in the regulation of phas expression. Experimental evidence was obtained by in vivo and in vitro footprinting studies that the lack of transcriptional expression in vegetative tissues is stringently maintained by a rotationally and translationally positioned nucleosome over the three-phased TATA boxes of the phas promoter (Li et al., 1998), each of which contributes to its high level of expression (Grace et al., 2004). A significant finding was that, although transcription from the phas promoter is not inducible in callus or vegetative tissues by the plant growth regulator abscisic acid (ABA) alone (Frisch et al., 1995), ectopic expression of a seed-specific transcriptional activator, Phaseolus vulgaris ABI3-like factor (ALF) from the quasiconstitutive cauliflower mosaic virus 35S promoter (Moravcikova et al., 2004), renders phas ABA-inducible in vegetative tissues (Li et al., 1999). Increased DNase I accessibility to the phas promoter in isolated nuclei was observed in the presence of ALF, but the TATA boxes became protected in the presence of both ALF and ABA, suggesting that chromatin remodeling facilitates occupancy by TATA binding protein (TBP) under those conditions. These observations provided evidence for a two-step process of phas activation in which the first step (potentiation) requires the presence of ALF and the second step (activation) is achieved by ABA acting through a signal transduction pathway. Placement of ALF expression under the control of an estradiol-inducible promoter (Zuo et al., 2000) permits analysis of the chromatin status over the phas promoter under three discrete conditions. To maintain the repressed state, no estradiol is supplied, so that ALF production is uninduced and no ABA is added. The potentiated state is attained by supplying estradiol and hence ALF, but no ABA. When both estradiol and ABA are
supplied, the phas promoter is transcriptionally active. This system permits the distinction of events related to the remodeling of nucleosome architecture over the promoter from the ABA-motivated recruitment of TBP and initiation of transcription.

Evaluation of the covalent histone modifications associated with the developmental stages and transcriptional status of eukaryotic promoters has verified the existence of an epigenetic code (Turner, 2000; Jenuwein and Allis, 2001), and rapid advances are being made in deciphering its roles in developmental processes of higher organisms (Margueron et al., 2005). Studies on the recruitment of specific factors or complexes by specific histone states are providing exciting insights into gene regulation. In plants, elegant studies on vernalization and control of flowering time have revealed that the chromatin status over FLOWERING LOCUS C (FLC) is influenced by three regulatory systems and controls downstream flowering-time integrator genes that in turn activate floral meristem identity genes (Boss et al., 2004; Putterill et al., 2004; He and Amasino, 2005). Among animal systems, characterization of histone modifications over the interferon-β (IFN-β) promoter after infection by Sendai virus is providing novel insights into how transcription is initiated (Agalioti et al., 2002).

A challenging question regarding chromatin dynamics is the fate of the nucleosome during transcriptional activation. Using a novel photochemical method for mapping the contacts of specific histone residues with DNA in the nucleosome before and after remodeling, Kassabov et al. (2003) demonstrated that, in addition to sliding nucleosomes, SWI/SNF displaces DNA off the octamer in a process that remodels 50 bp of DNA within 1 s. This concept appears to be in good agreement with histone changes seen here for the phas promoter.

In this work, we show that the three discrete conditions of the phas promoter are reflected in various arrays of chromatin modifications. In addition to the discrete separation of potentiation from activation, our system allows chronological studies that provide insight into the ordered recruitment of histone modifiers. Insight gained from these studies suggests the existence of close similarities between transcriptional activation of the phas and IFN-β promoters.

RESULTS

Inducible Potentiation and Activation System for phas Expression in Vegetative Tissues

The seed-specific activation of transcription from the phas promoter is a two-step process that includes ALF-mediated remodeling of its chromatin architecture (potentiation) and subsequent activation through an ABA-mediated signaling cascade (Li et al., 1999). In developing bean seeds, events associated with phas potentiation and activation are inseparable, as both ALF and ABA are present. To differentiate between these sequential events, an estrogen receptor–based inducible system (Zuo et al., 2000) was used (Figure 1A) to permit the ectopic expression of a hemagglutinin (HA) epitope–tagged ALF (HA-ALF) in leaves of Arabidopsis thaliana supertransformants. A triple HA tag sequence was fused 5′ to the ALF coding sequence (HA-ALF) and inserted downstream of a synthetic promoter containing the LexA operator to yield the inducible construct XVE-HA-ALF. XVE is a chimeric protein that contains the LexA DNA binding domain, and

Figure 1. Induced Ectopic Expression of HA-ALF and phas Activation in Leaves. 
(A) Scheme of the XVE-HA-ALF-inducible effector construct and the −1470phas-gus reporter construct present in 5′14HA-ALF Arabidopsis supertransformants.
(B) 12.5% SDS-PAGE (top) and protein gel blot analysis with anti-HA antibody of a parallel gel (bottom) of total protein (40 μg/lane) from leaves treated for 12 h with 25 μM estradiol (E), 25 μM estradiol plus 200 μM ABA (EA), or 200 μM ABA (A). M, protein size markers; U, uninduced control.
(C) Histochemical analysis of GUS expression in representative 5′14HA-ALF plantlets for each treatment. Bars = 1 mm.
the VP16 activation domain, and the regulatory region of the human estrogen receptor (Zuo et al., 2000). When leaves of transgenic Arabidopsis line 5’14HA-ALF (containing both the −1470phas-gus reporter and the XVE-HA-ALF effector constructs) are exposed to estradiol, the XVE transactivator protein is induced and binds to the LexA operator, driving ectopic expression of HA-ALF. The presence of HA-ALF poises (potentiates) the repressed phas promoter for transcriptional activation upon the addition of ABA.

To confirm that HA-ALF expression was dependent on ectopically supplied estradiol, leaves from 5’14HA-ALF plants were placed for 12 h in Murashige and Skoog (MS) medium (see Methods) containing 25 μM estradiol (E), 25 μM estradiol and 200 μM ABA (EA), or 200 μM ABA (A). Leaves exposed under identical conditions to medium lacking estradiol and ABA served as uninduced controls (U). Immunoblot analyses of total protein extracts confirmed that HA-ALF was produced only in leaves exposed to medium containing estradiol (Figure 1B). Histochemical staining for GUS expression verified that transcription from the phas promoter in leaves occurred only in the presence of both HA-ALF and ABA (Figure 1C, EA). These experiments confirmed the feasibility of separating the potentiated state of the phas promoter (as a result of HA-ALF expression) from the transcriptionally active state in leaf tissues through the use of an inducible expression system. This permitted detailed analyses of changes in histone modifications associated with each step of transcriptional activation of the phas promoter in leaves. Such separation of potentiation from activation is not practicable in seeds because of the presence of both ABI3 (the Arabidopsis homolog of ALF) and ABA. Furthermore, the very small size of developing Arabidopsis embryos greatly limits the amount of material suitable for characterization of chromatin status.

### Potentiation and Activation of phas Can Be Temporally Separated

To optimize the induction conditions for HA-ALF production and ABA activation of the phas promoter, phas-driven GUS production was followed. Rosette leaves from 3- to 4-week-old 5’14HA-ALF plants were subjected to estradiol (25 μM) and ABA (200 μM) treatment, and samples were collected for GUS staining at various time points (Figure 2A). GUS expression was initially detected after 4 h, and uniform histochemical staining in the leaves was obtained after 8 h. This finding indicated that potentiation resulting from HA-ALF production occurs within 4 h of estradiol addition.

In another set of experiments, leaves of 5’14HA-ALF plants were exposed to estradiol for 1 to 4 h to induce the production of HA-ALF, after which the estradiol was removed. ABA was added after the removal of estradiol (Figure 2B). Histochemical staining of leaves treated with estradiol for 4 h revealed that GUS was produced substantially earlier (1 h) than in leaves treated for only 1 h with estradiol. This finding suggests that the rate of GUS production is dependent on the amount of HA-ALF available. After 5 h of exposure to ABA, uniform GUS expression was observed for all treatments; unless specified otherwise, 8 h was chosen as the standard incubation time for all subsequent analyses.

**Figure 2. Activation of the phas Promoter.**

(A) Histochemical staining of leaves from the 5’14HA-ALF line at the indicated times after addition of 25 μM estradiol and 200 μM ABA.

(B) phas was potentiated by treatment of leaves from the 5’14HA-ALF line with 25 μM estradiol for 1 to 4 h. ABA was added to a final concentration of 200 μM after estradiol was removed at the indicated times. Histochemical-stained leaves are shown for the indicated times after ABA addition.
Nucleosomal Architecture of the *phas* Promoter during Potentiation and Activation

A rotationally positioned nucleosome over the three-phased TATA boxes in the *phas* promoter represses activation in vegetative tissue (Li et al., 1998). In transgenic tobacco (*Nicotiana tabacum*) leaves ectopically expressing ALF, DNase I hypersensitivity assays coupled with ligation-mediated PCR showed an increase in DNase I sensitivity over the TATA region of the *phas* promoter (Li et al., 1999). These assays also revealed a shift in the rotationally positioned nucleosome, commensurate with remodeling of the nucleosome at the *phas* promoter in the presence of ALF.

In this study, chromatin immunoprecipitation (ChIP) was used to determine the nucleosomal condition of the *phas* promoter upon potentiation and activation in leaves from 5′14HA-ALF plants under the four experimental regimes (U, E, EA, and A) (see Methods). DNA purified after immunoprecipitation was evaluated by PCR using primers targeting the proximal region of the *phas* promoter. The targeted 227-bp amplicon (Figure 3) includes four RY elements (the binding motif for ALF) (Carranco et al., 2004) and a G-box (ABA response element [ABRE]) (Ezcurra et al., 2000) that are essential for *phas* expression (Chandrasekharan et al., 2003a). As an internal control, primers targeting the 5′ end (133 bp) of *actin7* (McDowell et al., 1996) were used for PCR.

Histone H3 and H4 occupancy at the *phas* promoter was first determined by ChIP assays using antibodies against the N terminus of histone H3 and the C terminus of histone H3 or H4. Upon potentiation, the nucleosome present at the proximal region (−282 to −55) of the *phas* promoter was remodeled, as indicated by a decrease in histones H3 and H4 (Figure 3). The *phas* PCR signal was quantified by densitometry with normalization against the mock control, input DNA, and the *actin7* signal (see Methods) to yield an enrichment value. A relative enrichment (RE) value for each individual treatment (E, EA, or A) was calculated relative to the enrichment value for the uninduced control (U), and standard errors from repeated experiments were calculated (Figure 3, left side). The results of these experiments indicate that a decrease in histone–DNA interaction or displacement of histones from the *phas* promoter occurs during *phas* potentiation. A slight increase in the level of histones H3 and H4 was detected when the *phas* promoter was transcriptionally active, suggesting an increase in histone–*phas* interaction or the redeposition of histones in the *phas* chromatin.

Histone Acetylation Associated with *phas* Expression

In general, histone hyperacetylation is correlated with the permissive state of gene expression (Wade et al., 1997; Turner, 2000; Lusser et al., 2001). Using anti-acetyl histone H3 antibody in ChIP analyses, a slight increase in histone acetylation was detected during *phas* potentiation (E) and activation (EA) (Figure 4A). In yeast, using antibodies against the C terminus of histone H3 or H4, Reinke and Horz (2003) found that an apparent decrease in

Figure 3. Remodeling of *phas* Chromatin during Potentiation and Activation.

Leaves from 5′14HA-ALF plants were subjected to the four regimes described in the legend to Figure 1. ChIP assays were performed with antibodies against the N terminus of histone H3 (A), the C terminus of histone H3 (B), or the C terminus of histone H4 (C). Representative ChIP-PCR products for the 227-bp *phas* (−282 to −55; see diagram at top) and the 133-bp *actin7* amplicons are shown at right. The ethidium bromide–stained products were quantitated by densitometry, and the average RE values are shown as histograms at left. Error bars represent standard errors.
Histone acetylation level at the activated \textit{PHO5} promoter was attributable to a progressive loss of total histones. Normalization of the acetylation level with respect to histone occupancy revealed that histone acetylation at the activated promoter increased, rather than decreased. Similarly, we detected an apparent decrease in histone signals when \textit{phas} chromatin was potentiated and activated (Figure 3). Normalization of the RE values for diacetylated histone H3 (Figure 4A, gray bars) against the respective RE values shown in Figure 3B for histone H3 revealed a substantial increase in histone diacetylation (RE = 5.11 ± 0.91) upon potentiation and activation (RE = 2.85 ± 0.38) of the \textit{phas} promoter. A slight increase in diacetylation of histone H3 was also detected in the presence of ABA alone (RE = 1.1 ± 0.16).

\textbf{Figure 4.} Histone Acetylation and Methylation Associated with \textit{phas} Expression.

Representative PCR results showing the 227-bp \textit{phas} amplicon (−282 to −55; top) from ChIP assays using various histone antibodies: anti-diacetyl H3 (K9/K14) (A); anti-acetyl H3-K9 (B); anti-acetyl H3-K14 (C); anti-hyperacetylated H4 (K5/K8/K12/K16) (D); anti-acetyl H4-K5 (E); anti-acetyl H4-K12 (F); anti-dimethyl H3-K4 (G); anti-trimethyl H3-K4 (H); and anti-dimethyl H4-K20 (I). \textit{Actin7} was amplified as an internal control for all PCRs. Average RE values are shown as histograms at bottom (gray bars). Respective RE values for histone modification levels were normalized against the RE values shown in Figure 3 for histone H3 or H4 (black bars). Error bars represent standard errors.
To further characterize histone acetylation at specific Lys residues upon phas expression, histone antibodies against either H3-K9 or H3-K14 acetylation were used. Interestingly, H3-K9 acetylation was significantly enriched during phas potentiation and decreased when phas was actively transcribing (Figure 4B). Normalization of the PCR signals gave RE values of 10.3 ± 3.46 and 3.69 ± 0.31 for the potentiated and activated states, respectively, compared with the repressed state (Figure 4B, gray bars). Although these values revealed the change in H3-K9 modification, additional normalization of the data for histone occupancy (Figure 4B, black bars) yielded RE values for H3-K9 of 27.8 ± 10.8 (potentiation) and 6.21 ± 0.6 (activation), accentuating the dramatic nature of these modifications in the early stages of transcriptional expression. In contrast with the situation for H3-K9, a decrease in H3-K14 acetylation was detected upon phas potentiation (RE = 0.36 ± 0.22). By correcting the RE value for H3-K14 acetylation against that for histone H3 occupancy, it became evident that H3-K14 acetylation increased considerably when phas was actively transcribing (RE = 2.42 ± 1.13), although no difference in the level of acetylation was observed between the repressed and potentiated (RE = 0.98 ± 0.59) phas chromatin (Figure 4C, black bars).

Unlike histone H3 acetylation, the data shown in Figure 4D revealed that histone H4 was hyperacetylated only during phas activation (gray bars; RE = 1.42 ± 0.18). However, correction of the histone H4 hyperacetylation RE value for histone H4 occupancy revealed an apparent increase in acetylation during phas potentiation (RE = 1.71 ± 0.13) and activation (RE = 2.1 ± 0.26). It is clear from these findings that, in addition to an input DNA control and an internal control, such as actin7, compensation for changes in histone occupancy is important for correct interpretation of histone modification during gene expression by ChIP analysis. Using antibodies against specific Lys residues on histone H4, a marked enrichment of acetylation at H4-K5 was detected during phas activation (RE = 6.4 ± 3.52) but not potentiation. However, results from leaves treated with ABA alone (A) also showed an enrichment of H4-K5 acetylation (RE = 8.08 ± 5.46) compared with the uninduced control (U) (Figure 4E). Normalization of RE values with those for histone H4 occupancy revealed a slight increase during phas potentiation (black bars; RE = 2.13 ± 0.18) and a significant increase in H4-K5 acetylation when phas was activated (RE = 9.46 ± 5.2) as well as in the presence of ABA alone (RE = 7.68 ± 5.19). In contrast with H4-K5 acetylation, a slight increase in acetylation of histone H4 at Lys-12 was observed during phas potentiation (Figure 4F; RE = 1.37 ± 0.31). When corrected for histone H4 occupancy, it clearly showed that H4-K12 is acetylated upon phas potentiation (RE = 3.25 ± 0.37) and deacetylated (RE = 0.93 ± 0.37) when phas was actively transcribing (Figure 4F, black bars).

Therefore, by temporally separating phas potentiation from activation, we discovered that the Lys residues of histones H3 and H4 are not globally hyperacetylated during phas expression. Rather, there is a stepwise acetylation and deacetylation of histones at specific Lys residues as phas is induced to its potentiated state and as it progresses to its activated state.

Histone Methylation Status during phas Expression

Lys residues in histone tails can be monomethylated, dimethylated, or trimethylated, and Arg residues can be dimethylated (both asymmetrically and symmetrically) or monomethylated (Lachner and Jenuwein, 2002; Bannister and Kouzarides, 2004; Tariq and Paszkowski, 2004). Methylation of specific Lys or Arg residues can lead to either repressive or permissive states of gene expression (Kouzarides, 2002). To elucidate the histone methylation status associated with phas expression, ChIP analyses were performed using antibodies against methylated Lys residues H3-K4, H3-K9, or H4-K20 under the ascribed experimental treatments (U, E, EA, and A) of 5’14HA-ALF plants. ChIP using dimethyl or trimethyl H3-K4 antibodies showed that both dimethylation and trimethylation of H3-K4 are associated with active transcription from the phas promoter (Figures 4G and 4H). However, an increase in H3-K4 dimethylation was also detected in the presence of either estradiol (RE = 3.28 ± 0.27) or ABA (RE = 4.21 ± 2.85) alone. Nevertheless, these data are in accord with the general observation that methylation at histone H3 at Lys-4 is associated with gene activation (Strahl et al., 1999; Litt et al., 2001; Ng et al., 2003a).

Although H4-K20 was dimethylated in uninduced leaves or leaves treated with ABA alone (RE = 1.45 ± 0.59), a slight decrease in H4-K20 dimethylation was detected during phas potentiation (RE = 0.7 ± 0.18) and transcriptionally active phas chromatin was found to be devoid of dimethylated H4-K20 (RE = 0.36 ± 0.09) (Figure 4I, gray bars). These findings suggest that dimethylation of histone H4 at Lys-20 does not impede chromatin remodeling or phas potentiation, even though it has been imputed to function in general gene silencing (Karachentsev et al., 2005). Similar results were obtained when the decrease in histone H4 occupancy was included in calculating the RE value for dimethylated H4-K20 level during phas activation (Figure 4I, black bars; RE = 0.52 ± 0.14). By contrast, an enrichment of H4-K20 dimethylation was revealed at the potentiated phas chromatin (RE = 1.67 ± 0.44) after the normalization with histone H4 occupancy. Methylation of histone H3-K9 is also generally associated with gene silencing and heterochromatin formation through the recruitment of heterochromatin protein 1 (Bannister et al., 2001; Mutskov and Felsenfeld, 2004). To determine the methylation status of H3-K9 at the phas chromatin, antibodies (from either Upstate or Abcam; see Methods) against dimethylated H3-K9 were used in ChIP analyses. However, we were unable to obtain reproducible results for H3-K9 methylation status at the phas promoter with either antibody. Therefore, it remains to be determined whether H3-K9 methylation is a characteristic of repressed phas chromatin.

In the presence of both HA-ALF and ABA, RNA polymerase II (Pol II) is recruited to the poised phas promoter, as shown by ChIP assays using antibody against the C-terminal domain repeats of yeast RNA Pol II (Figure 5). This finding shows that Pol II is not recruited to the remodeled phas chromatin in the presence of ALF alone, in agreement with previous evidence (Li et al., 1999) that components of the ALF signaling cascade are required for its recruitment and subsequent activation of transcription. In addition, leaves treated with ABA alone showed no association of RNA Pol II with the phas promoter, confirming that
remodeling by ALF is a prerequisite for access to the phases chromatin by RNA Pol II.

### Temporal Profile of Histone Modifications during phases Potentiation and Activation

It is now evident that chromatin architecture is dynamic, and considerable interest exists in elucidating the chronological order of chromatin changes associated with promoter activation (Agalioti et al., 2000; Cosma, 2002). The inducible system used in these studies provided an excellent opportunity to investigate the temporal order of alterations in histone modification associated with the potentiation and activation of the phases promoter. The procedure shown in Figure 2A was modified so that only estradiol was added at the beginning of the experiment and hence only potentiation (chromatin-remodeling) events could occur. As shown in Figure 6A, HA-ALF transcripts were detected by RT-PCR within 1 h after estradiol addition, increased in abundance over the next 3 h, then remained at similar levels for at least 5 h after estradiol was removed. H3-K9 acetylation was evident within 1 h of estradiol addition and was dramatically increased 1.5 h after the leaves were exposed to estradiol (Figure 6C). This is in accord with the finding that acetylation of H3-K9 is associated with phases potentiation (Figure 4B). Although HA-ALF production was not detected by protein gel blot analysis until 3 h after estradiol induction (Figure 6B), this probably reflects the relative insensitivity of this assay. In marked contrast with the early onset of H3 acetylation at Lys-9, only background (or nonspecific interaction) levels were detected for trimethylated H3-K4 throughout the potentiation step.

Transcriptional activation of the potentiated phases promoter was achieved by the addition of ABA to the incubation medium. A substantial increase in trimethylated H3-K4 was evident during the first 3 h after exposure of the leaves to ABA, and the signal continued to intensify for another 2 h (Figure 6C). By contrast, acetylation at H3-K9 decreased after the exposure to ABA. The appearance of gus transcripts within 1 h of ABA addition and their attainment of a steady state level during the subsequent 2 h (Figure 6A) reflect the completion of architectural remodeling of the phases promoter. GUS accumulation was detected by fluorometric analysis 3 h after the initiation of mRNA production and at 4 h by histochemical analysis (Jefferson et al., 1987) (Figure 6B).

### DISCUSSION

Epigenetic control via histone modifications is now known to play a significant role in gene expression and development (Schneider et al., 2004; Margueron et al., 2005). It has been shown in many systems that histone H3 and H4 hyperacetylation, H3-K9 acetylation, H3-K4 methylation, and H3-S10 phosphorylation are involved in active gene expression (Loidl, 2004), whereas hypoacetylated histones as well as H3-K9, H3-K27, and H4-K20 methylation are generally associated with gene silencing (Bender, 2004; Craig, 2005). In contrast with the wealth of information from animal and yeast systems, the relationship between chromatin dynamics and development has been investigated for only a few plant genes (Hsieh and Fischer, 2005).

The phases promoter, together with the GUS reporter, provides an excellent system for studying the dynamic changes in histone modification that accompany the transition of a higher eukaryotic promoter from a transcriptionally silent to a highly active state. The use of estradiol-inducible production of HA-ALF in Arabidopsis leaves allows precise analysis of these changes in chromatin status for the phases promoter as it is remodeled from its repressed form and is poised (potentiated) for transcriptional activation. Exposure of the potentiated promoter to ABA induces a second set of changes in nucleosomal architecture that result in transcriptional activation. Elucidation of the dynamic histone modification changes at the phases chromatin will thus provide insight into the identification of potential interactions between various chromatin modifiers and ALF as well as other components in the ABA signaling cascade throughout seed maturation in plant development.

### Histone Status of Repressed phases Chromatin

Histone H4 is dimethylated at Lys-20 when the phases chromatin is repressed from transcriptional activation, in accord with the finding that H4-K20 methylation functions in gene repression (Sarg et al., 2004; Karachentsev et al., 2005). In Drosophila, newly synthesized histones H3 and H4 are acetylated at K14/K23 and K5/K12, respectively, before their deposition into chromatin (Sobel et al., 1995). In yeast, identical acetylated histone isoforms were found in the replication-coupling assembly factor subunits that are involved in nucleosome reformation during DNA replication or repair (Tyler et al., 1999). The absence of H4-K5 acetylation at the repressed, phases chromatin may reflect a turnover...
of this acetyl group after the deposition of histones into the chromatin (Pesis and Matthews, 1986; Sobel et al., 1994). In the presence of ABA, H4-K12 showed a decrease in acetylation, suggesting its presence at the repressed *phas* chromatin. Thus, although this modification may have a structural role in *phas* chromatin architecture, its presence is not sufficient to weaken histone–DNA interaction and establish a permissive state for *phas* expression.

**Remodeling of *phas* Chromatin during Potentiation**

In plants, the onset of intense seed storage protein accumulation requires the presence of a B3 domain transcription factor such as ALF (in bean), ABI3 (in *Arabidopsis*), or VP1 (in *Zea mays*). These B3 factors are generally described as being plant-specific but are now known to include a region that has substantial similarity to the structure of the noncatalytic DNA binding domain of the restriction enzyme EcoRII (Yamasaki et al., 2004). We have previously shown that ectopic expression of ALF in tobacco leaves potentiates the normally stringently seed-specific *phas* promoter for transcriptional expression (Li and Hall, 1999). Similarly, ectopic expression of ABI3 in leaves, together with ABA, permits strong expression from the *phas* promoter in vegetative tissues of *Arabidopsis* (Ng et al., 2004). It thus appears that these factors are capable of recognizing the *phas* promoter in its nucleosomal architecture and of recruiting remodeling complexes that yield transcriptionally active chromatin over this promoter.

The inducible system used here, together with the increasing array of specific antibodies available for ChIP assays, permits identification of the changes and coordination in histone modifications associated with *phas* remodeling and can be expected to yield insights into the protein complexes involved. The decrease in histones H3 and H4 associated with *phas* promoter

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**Figure 6.** Temporal Changes in Histone Modification during Potentiation and Activation.

After 4 h of induction with 25 μM estradiol, leaves from 5′14HA-ALF plants were washed with water (to remove estradiol) and then placed in medium containing 200 μM ABA.

(A) RT-PCR analyses of HA-ALF, gus, and EF1α (control) transcripts.

(B) Recombinant HA-ALF detection using anti-HA antibody on a protein gel blot (top) and histochemical and fluorometric analyses for GUS expression (bottom).

(C) *phas* PCR products from ChIP assays using antibodies against acetylated H3-K9 and trimethylated H3-K4. 4-MU, 4-methylumbelliferone.
potentiation, as detected by ChiP analyses, suggest histone displacement from the promoter but could alternatively reflect a decrease in histone–DNA interaction, leading to less efficient cross-linking in the ChiP assay. Evidence favoring both possibilities exists. Hyperacetylation of histones over the activated PHOS promoter in Saccharomyces cerevisiae has been reported to result in the loss of histone contact and complete unfolding of nucleosomes (Reinke and Horz, 2003; Boeger et al., 2004). In an impressive study in yeast, Lee et al. (2004) showed a depletion in nucleosome occupancy at transcriptionally active regions throughout the genome. However, in a similarly impressive study, Kassabov et al. (2003) showed that SWI/SNF is engaged in a directional unwrapping of DNA from the edge of the nucleosome, resulting in the formation of a more accessible DNA loop during gene activation. This, and earlier work showing cooccupancy of transcriptional activators and histones for the HIV-1 enhancer (Steger and Workman, 1997), provide evidence that histones are not completely displaced when chromatin is being remodeled.

**Histone Modifications Associated with phas Activation**

As the ALF-potentiated phas chromatin progresses to its activated state in the presence of ABA, a transition phase can be discerned. This is characterized by an increase in histones H3 and H4, a gradual decrease in acetylated H3-K9, and increases in trimethylated H3-K4, acetylated H3-K14, and H4-K5 associated with the phas promoter. The increase in histones at the activated phas chromatin may reflect a simple restoration of histone–DNA interaction or a repositioning of histones from the potentiated phas chromatin. The observation that H4-K20 dimethylation is lost only upon phas activation suggests another possibility: that histone H3 displacement and replacement occurs during phas activation, putatively through a replication-independent nucleosome assembly pathway (Ahmad and Henikoff, 2002; Workman and Abmayr, 2004). It is possible, but not experimentally shown, that, as in Drosophila (McKittrick et al., 2004), histone H3.3, rather than H3, is the replacement histone. It is conceivable that the loss of H4-K20 dimethylation results from enzymatic demethylation. In this regard, a Lys-specific demethylase 1 was recently identified (Shi et al., 2004), but this enzyme targets exclusively to monomethylated or dimethylated H3-K4, and enzymes capable of removing other epigenetic methylation marks (methylated H3-K9, H3-K27, and H4-K20) remain unknown (Sarma and Reinberg, 2005).

**Similarities in Histone Code Functions among Eukaryotic Systems**

In agreement with the general observation that histone hyperacetylation is coupled with the permissive state of gene expression (Wade et al., 1997; Turner, 2000; Lusser et al., 2001), our results revealed an increase in histone H3 diacetylation (at Lys-9 and Lys-14) and histone H4 acetylation (hyperacetylation, or specific acetylation at Lys-5) when phas was potentiated and activated. Analysis of H3-K9, H3-K14, and H4-K12–specific acetylation revealed that both H3-K9 and H4-K12 acetylation were primarily associated with phas potentiation rather than activation. By contrast, acetylation at H3-K14 was found to be enriched only upon phas activation. A similar temporal order of histone modifications is associated with the initiation of transcription from the IFN-β promoter after infection by Sendai virus, in which H3-K9 acetylation commences 3 to 5 h after infection and persists throughout the time course of virus infection (Agalioti et al., 2002).

In the IFN-β system, an early step in transcriptional activation is the formation of an enhanceosome (Merika and Thanos, 2001). This complex contains three transcription factors (NF-κB, IRFs, and ATF-2/c-Jun) and HMG I(Y), an architectural protein that binds to specific sites in the nucleosome-free enhancer DNA, altering its topology and decreasing the free energy for activator binding (Falvo et al., 1995). In the phas system, ALF is known to be required for nucleosome remodeling (Li et al., 1998, 1999) and binds to RY motifs (Carranco et al., 2004) present within a 68-bp seed-specific enhancer sequence (van der Geest and Hall, 1996); it thus appears to be functionally similar to the architectural protein of the human IFN-β system.

In their silent state, the TATA regions of both the phas and IFN-β promoters are masked by a nucleosome, preventing access by basal transcription factors. By analogy to the IFN-β system, targeting of ALF to the RY motifs within the phas promoter can be predicted to lead to the formation of an enhanceosome, resulting in the recruitment of chromatin modifier(s) that contain histone acetyltransferase activity. Acetylation of H3-K9 is an early event in transcriptional activation of the phas promoter, and it is logical to speculate that, as for IFN-β, this event facilitates the recruitment of SWI/SNF by the enhanceosome. Subsequent remodeling of the nucleosome over the phas TATA region through interaction of bromodomains within the SWI/SNF complex with the acetylated histone N termini (Agalioti et al., 2002) would permit access by TFIID (Li et al., 1998), setting the stage for the establishment of the basal transcription complex. In the IFN-β system, the onset of H3-K14 acetylation correlates precisely with TBP recruitment and the initiation of transcription (Agalioti et al., 2002). Activation of the phas promoter is dependent on the addition of ABA and, as for the IFN-β promoter, is commensurate with acetylation of H3-K14. In addition to H3-K14 acetylation, activated phas chromatin is enriched with trimethylated H3-K4.

In S. cerevisiae, dimethylated H3-K4 is associated with both active and inactive euchromatic genes, whereas H3-K4 trimethylation results in active transcription (Santos-Rosa et al., 2002). The yeast SET1 complex was the first H3-K4 methyltransferase to be identified (Briggs et al., 2001). It targets the 5' portion of active mRNA coding regions through interactions with the Pol II–associated factor 1 (PAF1) complex and the RNA Pol II complex (Krogan et al., 2003; Ng et al., 2003b). The yeast PAF1 complex consists of Paf1, Ctr9, Leo1, Cdc73, and Rtf1 (Krogan et al., 2002; Squazzo et al., 2002). Arabidopsis relatives of Paf1, Ctr9, and Leo1 were identified as ELF7, ELF8, and VIP4, respectively (Zhang and van Nocker, 2002; He et al., 2004). These loci are involved in the FRIGIDA-mediated trimethylation of H3-K4 at the FLC chromatin in the winter-annual habit of Arabidopsis (He et al., 2004). These studies showed that members of the PAF1 complex in both yeast and plants share similar components in target gene regulation. As detected by ChiP assay in this study,
RNA Pol II is recruited to the *phas* chromatin in the presence of both ALF and ABA. By analogy, members of the PAF1 complex and H3-K4 methyltransferase (EFS in *Arabidopsis*; He and Amasino, 2005) may be recruited to the potentiated *phas* promoter through interaction with RNA Pol II in the presence of ABA. Such recruitment could initiate transcription, accompanied by changes in *phas* nucleosome architecture and histone modification.

**Model for Histone Modification Changes Associated with *phas* Expression**

The model proposed in Figure 7 summarizes the results obtained from ChIP analyses conducted in this study and places them in context with putative events leading to the activation of transcription from the *phas* promoter. It is known (Li et al., 1998) that during vegetative growth a rotationally positioned nucleosome is present over the three-phased TATA boxes of the *phas* promoter. Dimethylation of histone H4 at Lys-20 may contribute to the establishment of heterochromatic *phas* chromatin. During embryogenesis, the interaction of the transcription factor ALF (normally confined to developing embryos, but supplied ectopically in the leaf system described here) with RY elements of the *phas* promoter (Carranco et al., 2004) may lead to the recruitment of histone acetyltransferase through its acidic activation domain (Bobb et al., 1995). Acetylation of H3-K9 and H4-K12 thus weakens the histone–DNA interactions at the *phas* promoter or

![Figure 7](image-url)

**Figure 7.** Model Depicting the Sequential Events and Ordered Modification of Chromatin over the *phas* Promoter during Potentiation and Activation. Histone modifications associated with various *phas* promoter states are shown as symbols at right. Experimentally verified and putative pathways leading to *phas* activation are shown as blue (solid) and red (dotted) lines, respectively.

(A) In the repressed state during vegetative growth, the promoter is envisaged as being heterochromatic, with nucleosomes bearing dimethylated H4-K20.

(B) ALF-mediated potentiation of *phas* (1), possibly through recruitment of a complex with histone acetyltransferase (HAT) activity; H3-K9 and H4-K12 are acetylated. Histone modifications may recruit a chromatin-remodeling complex such as SWI/SNF, resulting in a decrease in histone–DNA interactions.

(C) Addition of ABA triggers the assembly of the ABA signaling cascade components (2) that interact with the ABRE within the *phas* promoter (3), leading to the recruitment of RNA Pol II and GTFs (4). New histone code modifications (H3-K4 trimethylation, H3-K14 and H4-K5 acetylation) are incorporated in the actively transcribed *phas* chromatin with the loss of histone H4-K20 dimethylation. During active *phas* transcription, histone displacement and redeposition of variant histones may take place that result in the deposition of new histone modifications at the *phas* chromatin. Although a marked increase in H4-K5 acetylation was evident during activation, a similar increase occurred when only ABA was added (see Figure 4E), suggesting that this modification may reflect events other than activation. The original repressive chromatin status of *phas* is restored at the end of seed maturation, and canonical histones are deposited into the *phas* chromatin through DNA replication during seed germination and vegetative growth.
constitutes a histone code for the recruitment of a chromatin-remodeling complex such as SWI/SNF. This, as a consequence, renders phas chromatin more accessible for the assembly of other factors and binding of the preinitiation complex as they are recruited through the ABA signaling cascade. In a recent article describing histone modifications over some 500 kb of the yeast genome at single-nucleosome resolution, Liu et al. (2005) raise the question of whether the changes in histone modifications play a permissive rather than an instructive role in the chain of events leading to transcription. In the case of the phas promoter, a dramatic increase in acetylation of H3 at Lys-9 was detected during potentiation, in which nucleosome remodeling occurs in the absence of transcription. This, together with DNase I footprinting data that also revealed ALF-mediated remodeling in the absence of transcriptional activation (Li et al., 1999), provides persuasive evidence that H3-K9 acetylation is instructive rather than permissive in the remodeling of this promoter.

The addition of ABA, or the onset of its presence in embryos, initiates the progression from potentiation to active transcription. Cooperative binding and interactions of ALF- and ABA-induced factors, such as ABIS (a basic leucine zipper transcription factor that binds ABRE; Nakamura et al., 2001), to the KY and ABRE motifs in the phas promoter thus permit the assembly of GTFs and RNA Pol II at the TATA regions. Upon transcription initiation, the repressive dimethylation mark at H4-K20 is removed by histone displacement from the phas chromatin, or by an unknown enzymatic reaction. Although not shown in our study, by analogy to results in yeast and Drosophila (Ahmad and Henikoff, 2002; Ng et al., 2003b), it remains possible that replication-independent nucleosome assembly is initiated and H3-K4 methylationtransferase (EFS in Arabidopsis) is recruited to RNA Pol II through the PAF1 complex when phas is activated. These events presumably lead to the deposition of replacement histone H3.3 in phas chromatin and specific modifications, including trimethylated H3-K4 and acetylated H3-K14 and H4-K5. A similar increase in H4-K5 acetylation also occurred when only ABA was added, suggesting that this modification may reflect events other than activation. The repressive phas chromatin state is restored once the levels of ALF and ABA decline at the end of seed maturation. We speculate that during seed germination, histone H3.3 present in the phas chromatin is replaced by canonical histone H3, together with modifications specifying repressed phas chromatin, through a replication-dependent nucleosome assembly pathway.

METHODS

Antibodies for ChIP Analyses

Antibodies used in ChIP assays were purchased from either Upstate or Abcam: anti-histone H3 N-ter (Upstate; 06-755), anti-histone H3 C-ter (Abcam; Ab1791), anti-histone H4 C-ter (Abcam; Ab10158), anti-acetyl-histone H3 (Upstate; 06-599), anti-acetyl-histone H3-K9 (Upstate; 07-352), anti-acetyl-histone H3-K14 (Upstate; 07-353), anti-dimethyl-histone H3-K4 (Upstate; 07-303), anti-trimethyl-histone H3-K4 (Upstate; 07-473), anti-hyperacetylated histone H3 (Upstate; 06-948), anti-dimethyl-histone H3-K9 (Upstate; 07-441), and anti-RNA Pol II CTD (Abcam; Ab5408).

Phas Construction

A triple-HA tag sequence (3xHA) was amplified by PCR from pMPY-3xHA (a kind gift from Michael P. Kladde, Texas A&M University) and cloned into a pGEM-T vector (Promega) to yield pGEM-T/3xHA. The 2.3-kb ALF coding region without the ATG start codon was amplified by PCR from pXVE-HisS-ALF (Chandrasekharan et al., 2003b) to incorporate flanking BssH and PacI restriction enzyme sites and cloned into a pGEM-T vector to yield pGEM-T/ALF. The ALF coding region was then released by BsmI and PacI digestion and fused 3’ to the 3xHA sequence in BsmI-PacI-digested pGEM-T/3xHA vector to give pGEM-T/HA-ALF. The HA-ALF fragment was then cloned into pER8 vector (Zuo et al., 2000) through Apal and PacI enzyme sites. The resulting construct, pER8/XVE-HA-ALF, was transformed into Agrobacterium tumefaciens strain GV3101 and used for Arabidopsis thaliana transformation to yield estrogen-inducible expression of HA-ALF.

Plant Transformation

Transgenic Arabidopsis (Columbia ecotype) seeds (line 5’14) containing –1470phas-gus (Chandrasekharan et al., 2003a) were germinated on MS agar medium (Sigma-Aldrich) containing 50 mg/L kanamycin (Sigma-Aldrich). Seeds were subjected to vernalization at 4°C for 2 d and grown at 22°C under a 16/8-h light/dark cycle. Agrobacterium-mediated transformation with pER8/XVE-HA-ALF of 4- to 5-week-old 5’14 plants was conducted with vacuum infiltration (Bechtold and Pelletier, 1998). Arabidopsis supertransformants (5’14HA-ALF) were selected by plating T0 seeds in MS agar containing hygromycin (25 mg/L) and kanamycin (50 mg/L). Transformants with a single homzygous insertion for both XVE-HA-ALF and –1470phas-gus transgenes were obtained through antibiotic selection and genomic blot analyses.

Estradiol and ABA Induction Conditions

The 5’14HA-ALF seeds were germinated in hygromycin (25 mg/L) and kanamycin (50 mg/L) selection medium, and rosette leaves from 3- to 4-week-old plants were collected and transferred to liquid MS medium containing 25 μM 17β-estradiol (Sigma-Aldrich) or 200 μM ABA (Sigma-Aldrich). Four independent treatments were performed: uninduced control without addition of estradiol and ABA (U); 25 μM estradiol alone (E); 25 μM estradiol and 200 μM ABA (EA); and 200 μM ABA alone control (A). Leaves were treated for 8 h with gentle agitation in the light unless specified otherwise. For chronological experiments in which estradiol was removed before ABA addition, leaves were exposed to MS medium containing 25 μM 17β-estradiol for 1 to 4 h, after which estradiol was removed by rinsing with running distilled water. The leaves were then exposed for the times indicated to MS medium containing 200 μM ABA.

Histochemical and Fluorometric Assays for GUS Activity

Histochemical staining and fluorometric analysis of GUS were performed with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid or 4-methylumbelliferyl-β-D-glucuronide, respectively, as substrates (Jefferson et al., 1987). For fluorometric analysis, GUS activity was calculated as pmoles of 4-methylumbelliferin per hour per microgram of protein.

Protein SDS-PAGE and Protein Gel Blot Analyses

After the specific induction treatment (U, E, EA, or A), total protein was extracted from leaves (100 mg) using an extraction buffer containing 200 mM Mops, pH 7.5, 200 mM KCl, 20% glycerol, 1 mM EDTA, and 3 mM DTT. Total protein was quantitated by the Bradford assay (Bio-Rad), and samples (40 μg of protein) were subjected to 12.5% SDS-PAGE. Benchmark prestained protein ladder (Invitrogen) was used as a molecular
marker for SDS-PAGE. Two parallel gels were prepared, one for Coomassie Brilliant Blue G 250 staining after electrophoresis, and the other for protein gel blot analysis. For protein gel blot analysis, total leaf proteins separated after SDS-PAGE were transferred to a polyvinylidene difluoride (Bio-Rad) membrane. Polyclonal HA antibodies (Covance Research Products) at 1:500 dilution were used for protein gel blots with chemiluminescent detection to verify the presence of HA-ALF in the samples.

**RT-PCR Analysis**

Total RNAs were extracted from leaves after various experimental treatments using TRIzol reagent (Invitrogen). DNase I–digested total RNAs were separated after SDS-PAGE were transferred to a polyvinylidene difluoride (Bio-Rad) membrane. Polyclonal HA antibodies (Covance Research products) were used for protein gel blot analysis. For protein gel blot analysis, total leaf proteins separated after SDS-PAGE were blotted and subsequently probed with a polyclonal anti-HA antibody. Immunoblots were generated with a Chemidoc system and analyzed using ImageJ software (National Institutes of Health). Normalized signal intensities were calculated by dividing the signal enrichment for the normalized histone signal by that for the uninduced control (U) and calculating the average of the results for the individual treatments (E, EA, or A) by that for the uninduced control (U) yielded the relative enrichment for each treatment. For Figure 4, the RE values for the various histone modifications were further normalized against the RE value for either histone H3 or H4 (Figures 3B and 3C) under high-salt (0.5 M) conditions for 6 h. For the input DNA control signals were determined similarly and, for each experimental condition, the signal from input DNA. Normalized actin7 control signals were performed using ImageJ software (National Institutes of Health). Normalized phas signals were obtained by subtraction of the mock (no antibody) control signal from the experimentally obtained value and correction for the signal from input DNA. Normalized actin7 control signals were determined similarly and, for each experimental condition, the signal from input DNA was used. The RE values for the various histone modifications were further normalized against the RE value for either histone H3 or H4 (Figures 3B and 3C) under the individual experimental regimes (U, E, EA, or A).

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers U28645 (ALF), U27811 (ACT7), and NM_100667 (EF1α).

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**REFERENCES**


Ordered Histone Modifications Are Associated with Transcriptional Poising and Activation of the phaseolin Promoter
Danny W-K. Ng, Mahesh B. Chandrasekharan and Timothy C. Hall
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DOI 10.1105/tpc.105.037010

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