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# Rapid and Reversible Light-Mediated Chromatin Modifications of Arabidopsis *Phytochrome A* Locus [C][W]

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**Recent genome-wide surveys showed that acetylation of H3K9 and H3K27 is correlated with gene activation during deetiolation of *Arabidopsis thaliana* seedlings, but less is known regarding changes in the histone status of repressed genes. Phytochrome A (phyA) is the major photoreceptor of deetiolation, and *phyA* expression is reversibly repressed by light. We found that in adult *Arabidopsis* plants, *phyA* activation in darkness was accompanied by a significant enrichment in the *phyA* transcription and translation start sites of not only H3K9/14ac and H3K27ac but also H3K4me3, and there was also moderate enrichment of H4K5ac, H4K8ac, H4K12ac, and H4K16ac. Conversely, when *phyA* expression was repressed by light, H3K27me3 was enriched with a corresponding decline in H3K27ac; moreover, demethylation of H3K4me3 and deacetylation of H3K9/14 were also seen. These histone modifications, which were focused around the *phyA* transcription/translation start sites, were detected within 1 h of deetiolation. Mutant analysis showed that HDA19/HD1 mediated deacetylation of H3K9/14 and uncovered possible histone crosstalk between H3K9/14ac and H3K4me3. Neither small RNA pathways nor the circadian clock affected H3 modification status of the *phyA* locus, and DNA methylation was unchanged by light. The presence of activating and repressive histone marks suggests a mechanism for the rapid and reversible regulation of *phyA* by dark and light.**

## INTRODUCTION

The nucleosomal organization of chromatin has been known to be an important element in the transcriptional regulation of eukaryotic genes. Core histone tails in nucleosomes can be reversibly modified through multiple posttranslational tags, including acetylation, methylation, ubiquitination or sumoylation of Lys residues, methylation of Arg residues, and phosphorylation of Ser and Thr residues (Strahl and Allis, 2000; Kouzarides, 2007). The impact of these modifications on the activation or repression of plant gene expression is being actively investigated on a genomic basis and for various signaling pathways (Charron et al., 2009; Roudier et al., 2009; Zhang et al., 2009; Zhou, 2009; Liu et al., 2010; Zhou et al., 2010).

After the first identification of *VERNALIZATION2* encoding a homolog of one of the Polycomb group proteins (Gendall et al., 2001) and *VERNALIZATION INSENSITIVE3* encoding a plant homeodomain protein (Sung and Amasino, 2004), chromatin modification has emerged as a critical regulatory mechanism in *Arabidopsis thaliana* vernalization. The molecular connection between vernalization and flowering prompted extensive studies on the chromatin status of the *FLOWERING LOCUS C* (*FLC*) locus (Kim et al., 2009). Activation of *FLC* expression prior to vernalization is associated with various activating histone marks such as histone H3 and H4 acetylation (He et al., 2003; Peng et al., 2006), H3K4 methylation (He et al., 2004), H3K36 methylation (Zhao et al., 2005), H2B monoubiquitination (Cao et al., 2008), and H2A.Z deposition (Deal et al., 2007), whereas vernalization-mediated *FLC* repression is associated with two repressive histone marks, H3K9 and H3K27 methylation (Bastow et al., 2004; Sung and Amasino, 2004).

Histone H3 modifications marked with both K4me3 (active) and K27me3 (repressive) are referred to as "bivalent domains" in embryonic stem cells (Bernstein et al., 2006), and such domains have been proposed to poise genes for rapid activation during differentiation or specific developmental stages. Both *FLC* and *FT* carry bivalent marks, H3K4me3 and H3K27me3, suggesting the role of H3K27me3 as a strong repression marker of the Polycomb complex (Finnegan and Dennis, 2008; Jiang et al., 2008).

Light (L) is among the most important environmental factors that impact plant development. When germinated in darkness (D), seedlings are etiolated, having elongated hypocotyls, closed cotyledons, and undifferentiated plastids. Upon exposure to L, seedlings undergo photomorphogenesis, during which hypocotyl elongation is inhibited, etioplasts differentiate into chloroplasts, chlorophyll is synthesized, and the cotyledons become expanded. These L-dependent morphological changes are underpinned primarily by changes in gene expression (Jiao et al., 2007), and it has been estimated that L alters the expression of ~20% of genes in the *Arabidopsis* genome (Jiao et al., 2005). The genome-wide reprogramming of gene expression during photomorphogensis suggests regulation at the chromatin level, including changes in histone modifications. Indeed, several reports have appeared on histone modifications of photosynthetic genes mediated by L. Chua et al. (2001, 2003) found that

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shoot-specific and L-induced accumulation of pea (*Pisum sativum*) *PetE* transcripts is associated with histone H3 and H4 acetylation in the *PetE* enhancer/promoter region. Genetic studies of *Arabidopsis* mutants deficient in histone acetyltransferase (TAF1/HAF2 and GCN5) and histone deacetylase (HDA19/HD1) provided evidence for the importance of histone acetylation/deacetylation in the expression of a number of photosynthetic genes (Bertrand et al., 2005; Benhamed et al., 2006). Subsequent genome-scale screening of target promoters bound by GCN5 identified early L-responsive genes similar to those targeted by HY5 (Lee et al., 2007; Benhamed et al., 2008). Recently, Charron et al. (2009) surveyed genome-wide distributions of four histone modifications (H3K9ac, H3K9me3, H3K27ac, and H3K27me3) during *Arabidopsis* seedling deetiolation and found acetylation of H3K9 but not H3K27 of the *HY5* and *HYH* loci upon L activation.

In contrast to genes that are induced by L, there is also a group of genes that are repressed by L. A notable example is *phytochrome A* (*phyA*), which encodes the major photoreceptor mediating far-red (FR) signaling. PhyA accumulates in D as the inactive, stable Pr form, but upon illumination it is converted to the active Pfr form (Sharrock and Clack, 2002), which is rapidly degraded by the COP1 E3 ligase (Seo et al., 2004). Along with phyA depletion in the L, *phyA* transcript levels are also strongly repressed by both FR and red (R) light (Cantón and Quail, 1999), suggesting L-mediated transcriptional regulation through both phyA and phyB photoreceptors. The negative regulation of *phyA* by L has been known for more than two decades; therefore, possible changes in *phyA* chromatin status during the D/L transition are of great interest.

Here, we confirmed that *phyA* transcript was repressed by L in a reversible manner and found that changes in transcript levels were correlated with alterations in specific histone marks. In the dark, acetylation of histones H3 (K9/14 and K27) and H4 (K5, K8, K12, and K16) and methylation of histone H3 (K4me3) were detected at the active *phyA* locus, whereas upon L treatment, increased H3K27me3 mark was associated with the repressed gene. The activating H3K4me3 mark and the repressive H3K27me3 mark were enriched around *phyA* transcription/translation start sites. The presence of these opposing marks enables rapid activation and inactivation of *phyA* in response to changing L conditions. Deacetylation of H3K9/14ac in the L was mediated by HISTONE DEACETYLASE19, and there is a coordinated behavior between H3K9/14ac and H3K4me3 suggesting cross-talk between these two marks. Finally, we found that that L-mediated *phyA* repression is regulated by chromatin remodeling without DNA methylation and without any participation of small RNA pathways.

## RESULTS

### *PhyA* Regulation in Adult Plants

We first used 2-week-old *Arabidopsis* plants to study global chromatin changes associated with D/L transitions. In general, there was an increase in histone H3 tail modifications in L compared with D, and notable changes are found with K9/K14ac, K4me2, K4me3, K27me3, and K36me3 (see Supplemental Figure 1 online). Time-course experiments during seedling deetiolation showed that the changes in H3 modification were rapid, as they can be detected even after only 1 h of L exposure; moreover, enrichments of both activating marks (e.g., K9/14ac and K4me3) and repressive marks (K27me3) were seen. These modifications reflect the sum of all histone H3 changes associated with both upregulation and downregulation of gene expression upon L treatment.

To investigate alterations in histone modifications specifically associated with *phyA* expression, we used 2-week-old plants grown under the same conditions. At the end of the L period (L1), plants were kept in D for 24 h (D1), transferred to L for 24 h (L2), and then returned to D for another 24 h (D2). Figure 1A shows that *phyA* transcript levels were clearly repressed by L in a reversible manner, confirming previous results (Cantón and Quail, 1999). The difference in transcript levels between D and L was approximately five to seven times.

*Arabidopsis* encodes only a single *phyA* gene of ~4 to 5 kb (Sharrock and Quail, 1989). We designed primers to interrogate 11 different regions of this *phyA* genomic locus from the promoter to the 3′ untranslated region (UTR; Figure 1B). Using polymerase II (Pol II)–specific antibodies, we detected specific enrichment of Pol II with region 3 in D as compared with L samples. Note that region 3 covers the genomic region just upstream of the ATG start codon. No Pol II enrichment was detected in regions 7 and 11, which correspond to the 3′ end of exon1 and the 3′ UTR, respectively. These results suggest that the reversible increase in *phyA* transcript levels in D is likely associated with Pol II–mediated transcription.

To investigate changes in chromatin status accompanying L repression and D activation of *phyA*, we performed chromatin immunoprecipitation (ChIP) using a battery of antibodies specific for modifications on H3 and H4. We focused on histone H3 changes in region 3 using regions 7 and 11 as negative controls. Figure 1D shows that D activation of *phyA* was accompanied by an enrichment (2.0- to 4.0-fold) of H3K4me3 and H3K9/14ac in region 3. This is in contrast to the general global increase of H3K4me3 and H3K9/14ac in L (see Supplemental Figure 1 online). The use of specific antibodies revealed increased levels of both H3K9ac and H3K14ac (Figure 1F). There was also a clear enrichment of H3K27ac and a corresponding depletion of H3K27me3 in D as compared with L samples. No significant and reproducible changes were detected with H3K4me2, H3K9me2, H3K9me3, and H3K36me3 levels. Small changes (<1.4-fold) in H3 marks in response to D and L were also seen in regions 7 and 11, but these were not considered to be significant (Figure 1E; see Supplemental Figure 2 online). We also examined possible H4 changes in region 3 and found only moderate enrichment (1.5- to 2.0-fold) of K5ac, K8ac, K12ac, and K16ac marks in D as compared with L (Figure 1G).

We compared changes of four key histone H3 marks (K4me3, K9/14ac, K27ac, and K27me3) over the 11 regions of the *phyA* locus between D and L samples. Figure 2 shows that D activation of *phyA* was clearly associated with an enrichment of H3K4me3 (four- to ninefold) in regions 2 and 3 and H3K9/14ac (two- to fourfold) in regions 3 and 4. Note that region 2 includes the *phyA* promoter, whereas region 3 spans the ATG translation start site. A strong enrichment of H3K4me3 (fourfold) but not H3K9/14ac

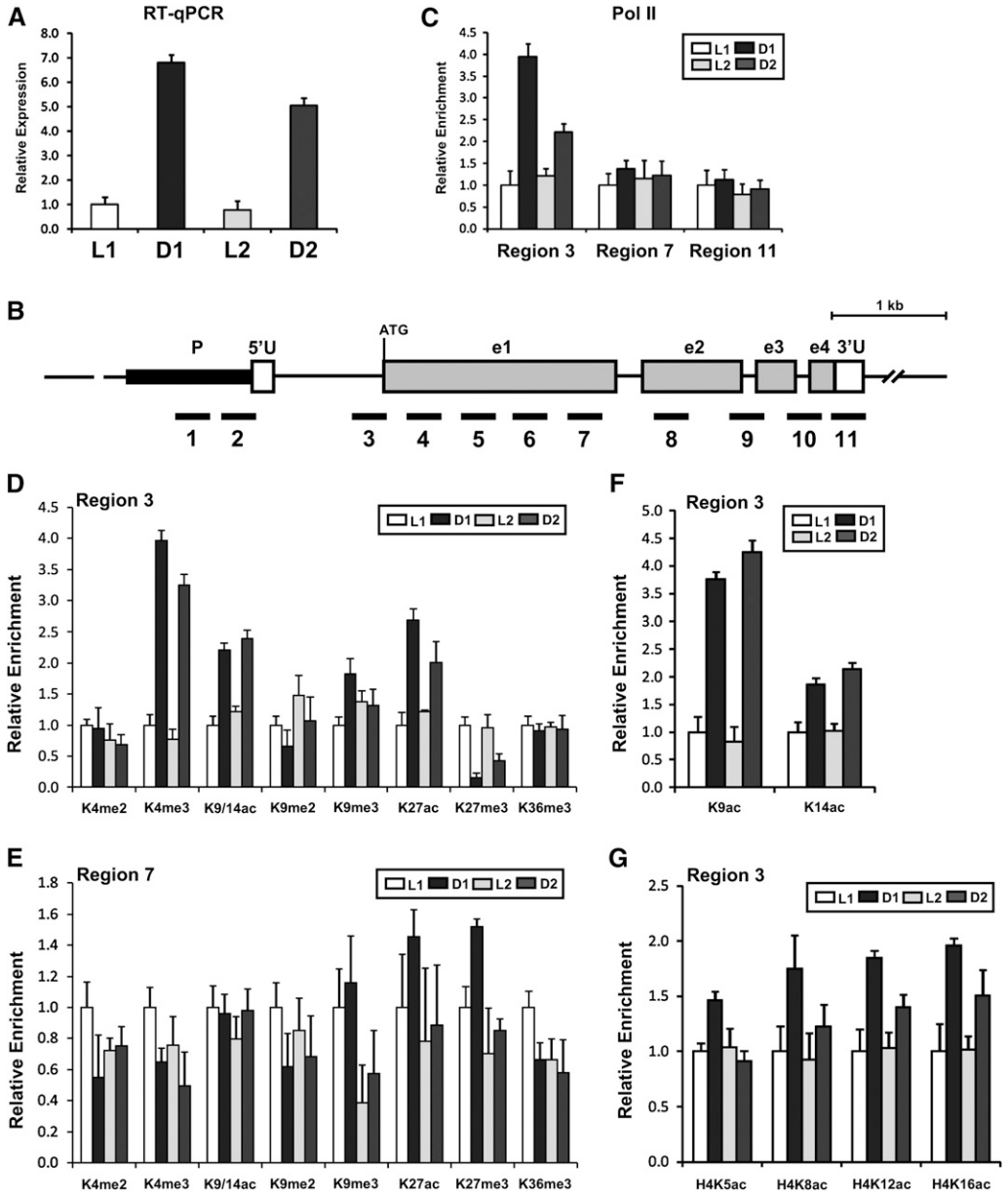


**Figure 1.** Changes of Histone Modifications on *phyA* in the D/L Transition.

**(A)** *phyA* expression levels in the D/L transition. Two-week-old *Arabidopsis* plants grown under long-day conditions (16 h of L/8 h of D) were harvested at the end of the L period (L1) and followed by D/L treatment for 24-h intervals (D1, L2, and D2). *PhyA* expression levels were determined by quantitative RT-PCR with a gene-specific primer set.

**(B)** Schematic diagram of *phyA* gene structure. Promoter (P), exons (e1, e2, e3, and e4), and 5′ UTR (5′U) and 3′ UTR (3′U) are represented by black, gray and white boxes, respectively. The amplification sites for ChIP analyses are indicated as numbers (1–11) with black bars below the map. ATG is the translation start site.

**(C)** RNA Pol II binding affinity on the *phyA* locus (regions 3, 7, and 11) during the D/L transition. The relative enrichment of RNA Pol II binding affinity was determined using anti-Pol II antibody with region-specific primers.

**(D)** and **(E)** Relative levels of histone H3 modifications (K4me2, K4me3, K9/14ac, K9me2, K9me3, K27ac, K27me3, and K36me3) at region 3 **(D)** and region 7 **(E)** in the D/L transition.

**(F)** Relative levels of histone H3 acetylation (K9ac and K14ac) in the D/L transition.

**(G)** Relative levels of histone H4 acetylation (K5ac, K8ac, K12ac, and K16ac) in the D/L transition.

Error bars indicate SD ($n$ = 3).

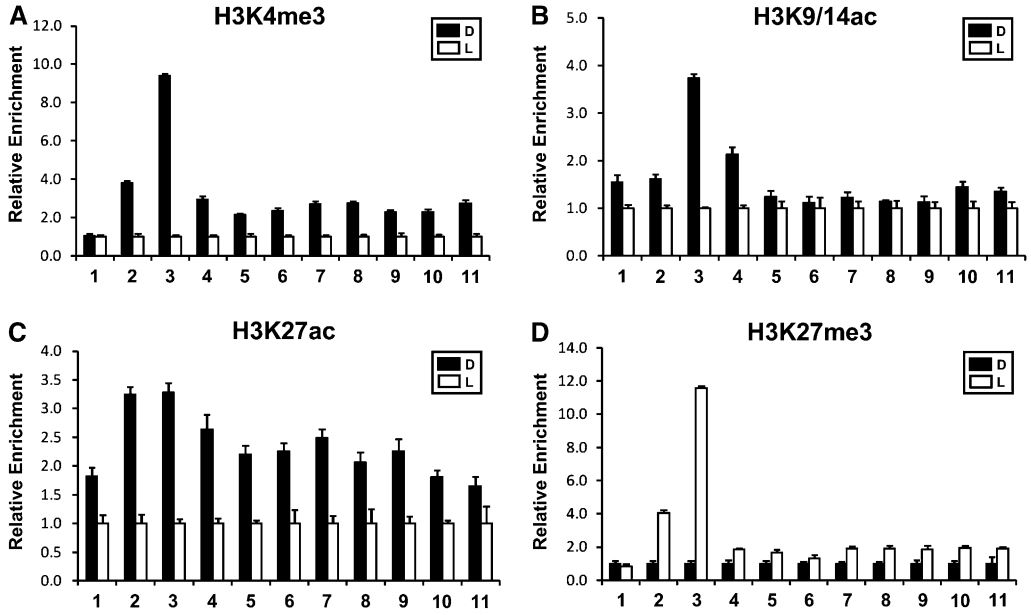


**Figure 2.** Enrichment of H3K4me3, H3K9/14ac, H3K27ac, and H3K27me3 at the *phyA* Locus.

Relative levels of H3K4me3 **(A)**, H3K9/14ac **(B)**, H3K27ac **(C)**, and H3K27me3 **(D)** at the *phyA* locus. Two-week-old *Arabidopsis* plants were grown under long-day conditions (16 h of L/8 h of D). Plants at the end of a L period were incubated in the D for 24 h (D) and then treated with white light for 24 h (L). The numbers on the *x* axis indicate the positions of the PCR-amplified sites described in Figure 1B. Error bars indicate SD ($n = 3$).

(1.5-fold) was also found in region 2, which includes the *phyA* promoter region. The enrichment of the H3K27ac mark (1.8- to 3.3-fold) was detected throughout all *phyA* regions but with a maximum in regions 2 and 3 (3.3-fold). By contrast, there was a 12-fold enrichment of H3K27me3 mark in region 3 and a fourfold enrichment of the same mark in region 2 upon silencing of *phyA* by L, indicating the importance of this repressive mark in *phyA* downregulation.

### *PhyA* Regulation during Deetiolation

Experiments with adult plants showed that the most prominent changes in the chromatin status of *phyA* during the D/L transition occurred around region 3 and, to a lesser extent, in region 2. As an important photoreceptor for seedling deetiolation, *phyA* transcript level is high in etiolated seedlings but rapidly declines upon exposure to L. We investigated whether chromatin changes would correlate with rapid *phyA* repression by L during seedling deetiolation. Figure 3A confirms a previous report (Cantón and Quail, 1999) that *phyA* transcripts accumulated in etiolated seedlings, but *phyA* was rapidly down-regulated upon irradiation with white light. However, this decrease in transcript level was reversed when seedlings were returned to D for 24 h (Figure 3A). By contrast, transcript levels of *phyB*, which was used as a negative control gene, were slightly increased in L as compared with D. The downregulation of *phyA* was accompanied by a decrease in Pol II association with region 3, suggesting reduced transcription (Figure 3B). The L-mediated repression of *phyA* was accompanied by a decrease in H3K4me3- and H3K9/14ac activating marks but an increase in H3K27me3 repressive mark (Figure 3C). These chromatin changes were readily detected after one hour of deetiolation, and they reached a steady state after 6 h of L. The changes were reversed when seedlings were returned to D for 24 h. No significant changes in these histone marks were seen in *phyB*, whose transcript levels only increased moderately in L. We have also checked Pol II enrichment and histone modifications in two different regions (regions 7 and 11 shown in Figure 1B) on the *phyA* locus. The enrichment of Pol II and histone modifications in region 7 showed similar patterns to those of region 3, but the fold change was less than 1.5 (see Supplemental Figures 3A and 3B online). No Pol II enrichment and changes of histone modifications were detected in region 11 (see Supplemental Figures 3A and 3C online).

### *PhyA* Regulation during D/L Diurnal Cycles

We also investigated *phyA* chromatin changes in 2-week-old plants grown under short-day conditions (8 h of L/16 h of D). At the end of a photoperiod, plants were kept in continuous D for another 24 h and samples were collected at 4-h intervals. Control experiments showed that transcript levels of *CCA1* decreased during the subjective L, as expected for this circadian regulated gene (Figure 4A). *PhyA* transcript levels were higher (~2.5-fold) in the D compared with the L period (Figure 4B). However, under prolonged D, *phyA* transcript levels continued to increase during the subjective L period. At the end of the second D period, *phyA* transcript levels were four to five times that at the beginning of the L period. These results clearly indicate that in contrast to *CCA1*, *phyA* is regulated by D/L only but not by the circadian clock. The increase in *phyA* transcript levels in D was accompanied by a three- to fourfold enrichment of H3K4me3 and H3K9/14ac marks in region 3 (Figures 4C and 4D), but no significant changes of

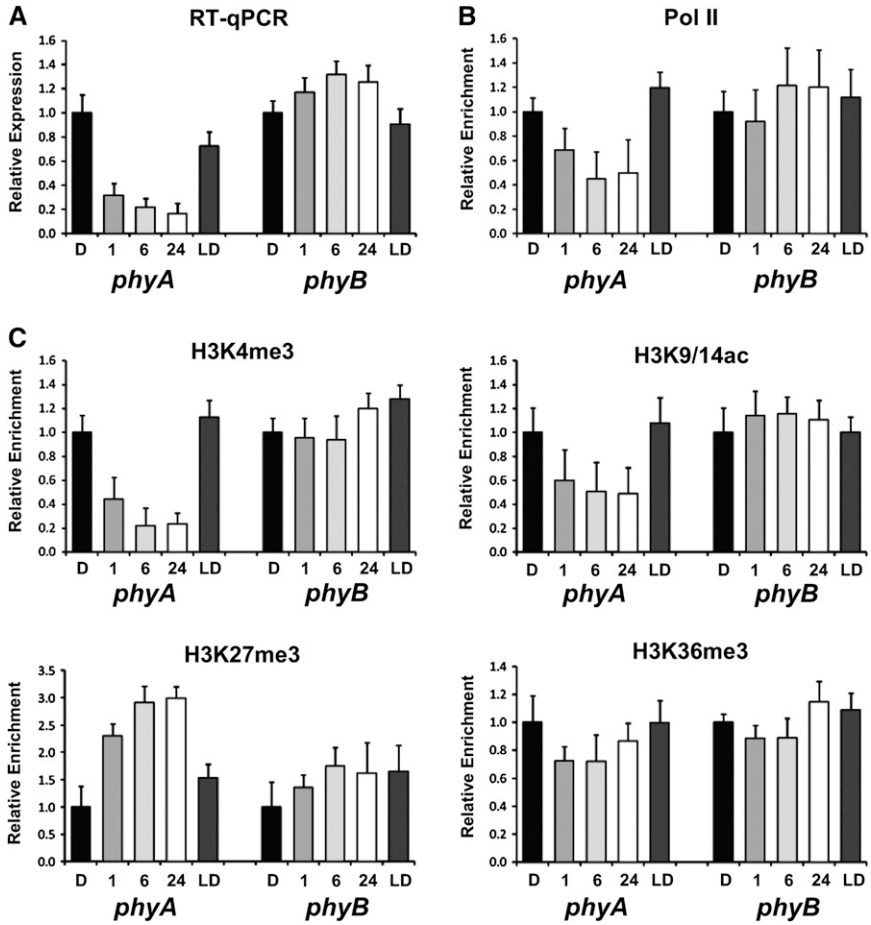


**Figure 3.** Changes of Histone Modifications on *phyA* during Deetiolation.

**(A)** Relative *phyA* and *phyB* expression levels during deetiolation. Four-day-old etiolated *Arabidopsis* seedlings (D) were treated with white light (100 μmol/m$^2$/s) as indicated (1, 6, and 24 h) and transferred to D (LD) for 24 h. Expression levels were determined by quantitative RT-PCR with gene-specific primer sets.

**(B)** Relative RNA Pol II binding affinity on *phyA* and *phyB* during deetiolation. The relative enrichment of RNA polymerase binding affinity at region 3 of the *phyA* locus was determined using anti-Pol II antibody.

**(C)** Relative levels of histone H3 modifications (K4me3, K9/14ac, K27me3, and K36me3) on *phyA* and *phyB* during deetiolation. The relative enrichment of histone H3 modifications at region 3 of the *phyA* locus was determined by ChIP assays using specific antibodies. Amplification sites of *phyB* for ChIP assay are similar to those of *phyA*.

Error bars indicate SD ($n$ = 3).

these modifications were seen in regions 7 and 11 (see Supplemental Figures 4A and 4B online).

### PhyB Signals *phyA* Chromatin Changes

The rapid repression of *phyA* expression has been reported to be regulated by both FR and R (Cantón and Quail, 1999). Figure 5A shows that *phyA* repression by R was blocked in the *phyB-9* mutant, consistent with the major role of the phyB photoreceptor in R responses. ChIP experiments showed that the continued high *phyA* expression in *phyB-9* in R was accompanied by a relative increase of H3K4me3, H3K9/14ac, and H3K27ac activating marks in the *phyA* region 3 in the *phyB-9* mutant as compared with the wild-type ecotype Columbia (Col-0; Figure 5B). Moderate increases (less than 1.5-fold) of these modifications were found in the *phyA* region 7 of the *phyB-9* mutant, but there were no differences in the *phyA* region 11 between the wild type and the *phyB-9* mutant (see Supplemental Figures 5A and 5B online).

### Deacetylation of H3K9/14 on the *phyA* Locus Depends on HDA19/HD1

The observation that *phyA* repression is accompanied by a decrease in acetylated H3K9/14 levels suggests that L mediates deacetylation of K9 and K14 on histone H3 tails. The *Arabidopsis* genome encodes a number of histone acetylases and deacetylases. At least three such enzymes are related to L responses, since mutants deficient in GCN5 and TAF1 have been shown to be hyposensitive to L whereas a mutant lacking in HDA19/HD1 is hypersensitive (Bertrand et al., 2005; Benhamed et al., 2006).

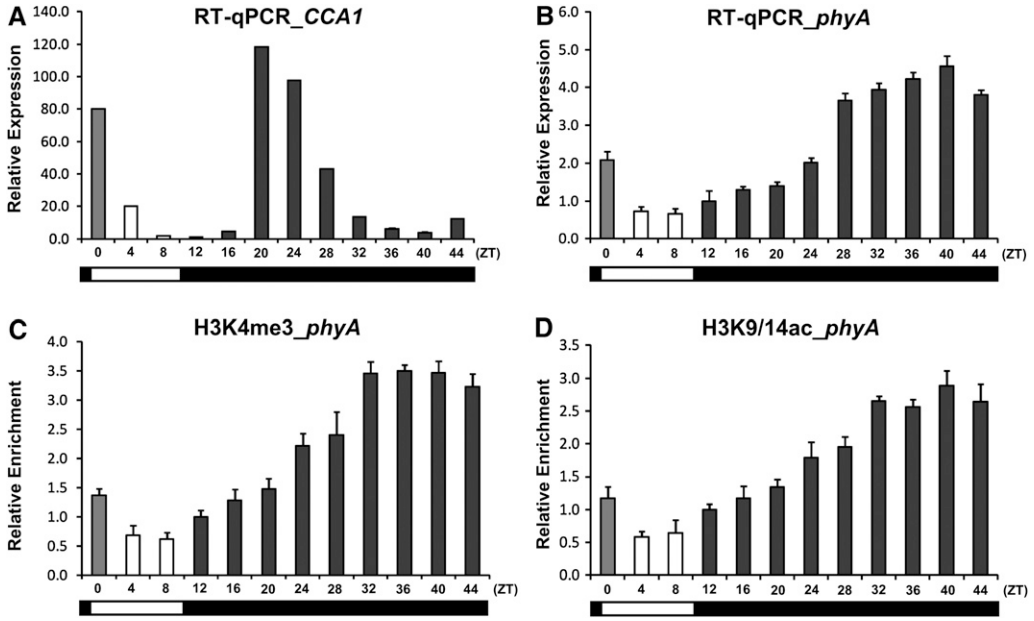


**Figure 4.** Changes of Histone Modifications on *phyA* during the Diurnal Cycle.

**(A)** and **(B)** Relative *CCA1* **(A)** and *phyA* **(B)** expression levels during the diurnal cycle. Two-week-old *Arabidopsis* plants were grown under short-day conditions (8 h of L/16 h of D), transferred to D for 24 h, and sampled at 4-h intervals. Expression levels were determined by quantitative RT-PCR with gene-specific primer sets.

**(C)** and **(D)** Relative levels of histone H3 modifications, K4me3 **(C)** and K9/14ac **(D)**, of *phyA* during the diurnal cycle. The relative enrichment of histone H3 modifications on region 3 of the *phyA* locus was determined by ChIP assays using specific antibodies.

Error bars indicate SD ($n$ = 3).

To investigate possible functions of these genes in regulating *phyA* expression, we compared *phyA* transcript levels of adult plants of *hda19/hd1* and *taf1* with those of the wild-type (Wassilewskija [Ws]) control. Figure 6A shows that L-dependent repression of *phyA* expression in *taf1* was similar to that in the wild-type (Ws) control but blocked in *hda19/hd1*. Consistent with this result, H3K9/14 acetylation was sustained at the D level in *hda19/hd1* even after 8 h of L exposure (Figure 6B), although in the wild type (Ws) and *taf1*, the H3K9/14 acetylation level declined to ~50% of the D level after the same L regimen. Further analysis showed that L-mediated demethylation of H3K4me3 occurred in *taf1* similar to the wild-type (Ws) control (Figure 6C), but surprisingly, the demethylation of H3K4me3 was blocked in *hda19/hd1* as well, which lacks HISTONE DEACETYLASE19. Our result shows that HDA19/HD1 but not TAF1 regulates *phyA* expression through deacetylation of H3K9/14. This finding confirms a previous report of an enrichment of H3K9ac associated with two photosynthetic genes in the *hda19/hd1* mutant compared with the wild type (Benhamed et al., 2006). No significant changes in H3 marks in regions 7 and 11 on the *phyA* locus were seen (see Supplemental Figures 6A and 6B online).

To see if HDA19/HD1 plays a similar role in *phyA* repression in young seedlings, we also investigated the responses of *hd1* and *taf1* during deetiolation, and essentially similar results were obtained. The kinetics of L-mediated *phyA* repression in *taf1* and the wild type (Ws) during deetiolation were similar, and after 6 h of L *phyA* transcript levels decreased to ~50% of the D level (Figure 6D). Similar time-course analysis shows that the L-dependent decline in *phyA* expression was significantly delayed although not totally arrested in *hda19/hd1*. After 6 h in L, *phyA* transcript level was ~70% of the D value. These differences in *phyA* expression in L between *hd1* and the wild type (Ws) and *taf1* were paralleled by differences in H3K9/14ac and H3K4me3 levels (Figures 6E and 6F). The less severe effect of the *hda19/hd1* mutation on *phyA* repression during deetiolation may be explained if there is functional redundancy contributed by other histone deacetylases at this stage of plant development.

## Dynamic Chromatin Changes Not Linked to Changes in DNA Methylation Status

We investigated possible linkage between D/L-mediated changes in *phyA* chromatin modifications and small RNA pathways. Supplemental Figure 7A online shows that a number of mutants (*rdr2*, *dcl3-1*, *ddc*, *nrpd1a-2*, and *nrpd1b-1*) deficient in small RNA pathway components were not significantly different from the wild-type control with respect to their D/L regulation of *phyA*. H3K4me3 changes accompanying D activation of *phyA* were clearly detected in *dcl3-1* and *nrpd1a-2* as in the wild-type control (see Supplemental Figure 7B online).

Chawla et al. (2007) previously reported transcriptional silencing of the endogenous *phyA* gene in transgenic plants carrying a transgenic fragment of *phyA*. In these plants, they observed methylation of certain *phyA* exonic regions, suggesting a correlation between *phyA* repression and DNA methylation. Therefore, we investigated whether the repression of *phyA* by

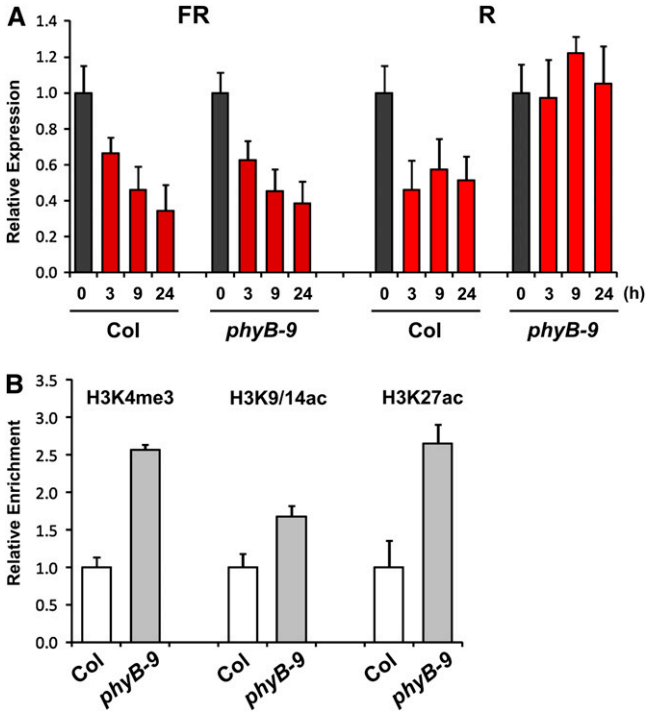


**Figure 5.** Enrichment of H3K4me3, H3K9/14ac, and H3K27ac at the *phyA* locus in the *phyB* Mutant Exposed to R.

**(A)** Relative *phyA* expression level in Col-0 and *phyB* mutant under FR or R. Four-day-old etiolated *Arabidopsis* seedlings were treated with FR (5 μmol/m$^2$/s) or R (20 μmol/m$^2$/s) as indicated (3, 9, and 24 h). Expression levels were determined by quantitative RT-PCR with a *phyA*-specific primer set.

**(B)** Relative levels of active histone H3 modifications (K4me3, K9/14ac, and K27ac) of *phyA* (region 3) in Col-0 and the *phyB* mutant after R (20 μmol/m$^2$/s) treatment for 24 h.

Error bars indicate SD ($n$ = 3).

[See online article for color version of this figure.]

L was accompanied by any DNA methylation. We performed sequencing of bisulfite-treated DNA collected from D and L samples and examined possible alterations in DNA methylation in six different regions of *phyA* (Figure 7; see Supplemental Figures 8 and 9 online). No significant changes in DNA methylation were detected between D and L samples in which *phyA* transcript levels were altered by six- to sevenfold (Figures 1A, D1 and L2, and 7; see Supplemental Figures 8 and 9 online). From these results, we conclude that reversible DNA methylation does not play a role in changes of *phyA* transcript levels during the D/L transition.

## DISCUSSION

PhyA plays an important role in early seedling development by mediating photomorphogenesis when germinated seedlings just emerge from the soil surface. Highly expressed in etiolated seedlings, this photoreceptor is rapidly downregulated by L both at the transcript (Cantón and Quail, 1999) and the protein levels (Seo et al., 2004). Although the L-mediated repression of *phyA* has been known for more than a decade, little is known regarding the molecular mechanisms of this repression. Here, we have focused on investigating chromatin changes on the *phyA* locus for three reasons: (1) phyA is the major photoreceptor for deetiolation; (2) its expression is repressed rather than activated by L; and (3) the *phyA* locus is longer than 5 kb, which allows more detailed investigations of changes in histone modifications along different genomic regions.

We show that the reversible activation and repression of *phyA* expression by D and L, respectively, is accompanied by reversible changes in H3 and H4 modifications of the *phyA* locus, particularly in regions surrounding the translation and transcription start sites. Because these histone modifications were seen when *phyA* transcript levels were altered under three different sets of conditions (16 h of L/8 h of D, exposure of etiolated seedlings to continuous L, and 8 h of L/16 h of D) irrespective of the growth stage of the plants, they must be related to the effects of L regulation on *phyA* expression. In contrast to previous work (Charron et al., 2009), we performed time-course studies to obtain information on the dynamics of expression changes and correlated these with changes in histone modifications.

### Activating Histone Marks

Four histone modifications, H3K4me3, H3K9ac, H3K14ac, and K3K27ac, were found to accompany *phyA* activation in D. Comparison of D and L samples showed that these marks were enriched by at least three- to ninefold. In addition, moderate enrichment (1.5- to 2.0-fold) of H4K5ac, H4K8ac, H4K12ac, and H4K16ac was also seen. H3K4me3, H3K9ac, and H3K14ac have been linked to active gene transcription in yeast and mammalian cells (Li et al., 2007) and more recently with activation of plant genes as well (Pfluger and Wagner, 2007; Kim et al., 2010).

With respective to L-responsive genes, activation of *HY5* and *HYH* during deetiolation is accompanied by a significant increase in H3K9ac (Charron et al., 2009), which is distributed over the entire transcribed region. By contrast, there is no detectable difference in H3K27ac levels associated with these two genes in D and L, and possible changes in H3K4me3 were not investigated. Notwithstanding the opposing mode of regulation, D activation of *phyA* was marked by an increase of not only H3K9ac but also H3K27ac. In addition, we observed an enrichment of H3K4me3 (10 fold), which was higher than that (three- to fourfold) found with H3K9/14ac and H3K27ac. Based on the relative enrichment, we suggest that H3K4me3 may play a more important role in *phyA* activation than the other two histone marks.

Charron et al. (2009) have raised a possible fundamental difference between yeast/mammalian and plant genes regarding the location of the activating H3K9ac mark. In yeast and mammals, H3K9ac is enriched around the gene promoter region, whereas in plants, H3K9ac and H3K27ac are found around the translation rather than transcription start site of active genes (Kim et al., 2008; Charron et al., 2009; Wang et al., 2009). Our results here are consistent with this finding, since not only H3K9/14ac but also the activating H3K4me3 mark are more enriched at the genomic region surrounding the translation start site (region 3), although significant changes were observed around the transcriptional start site as well (region 2). The case of H3K27ac deserves special comment. In contrast to the other

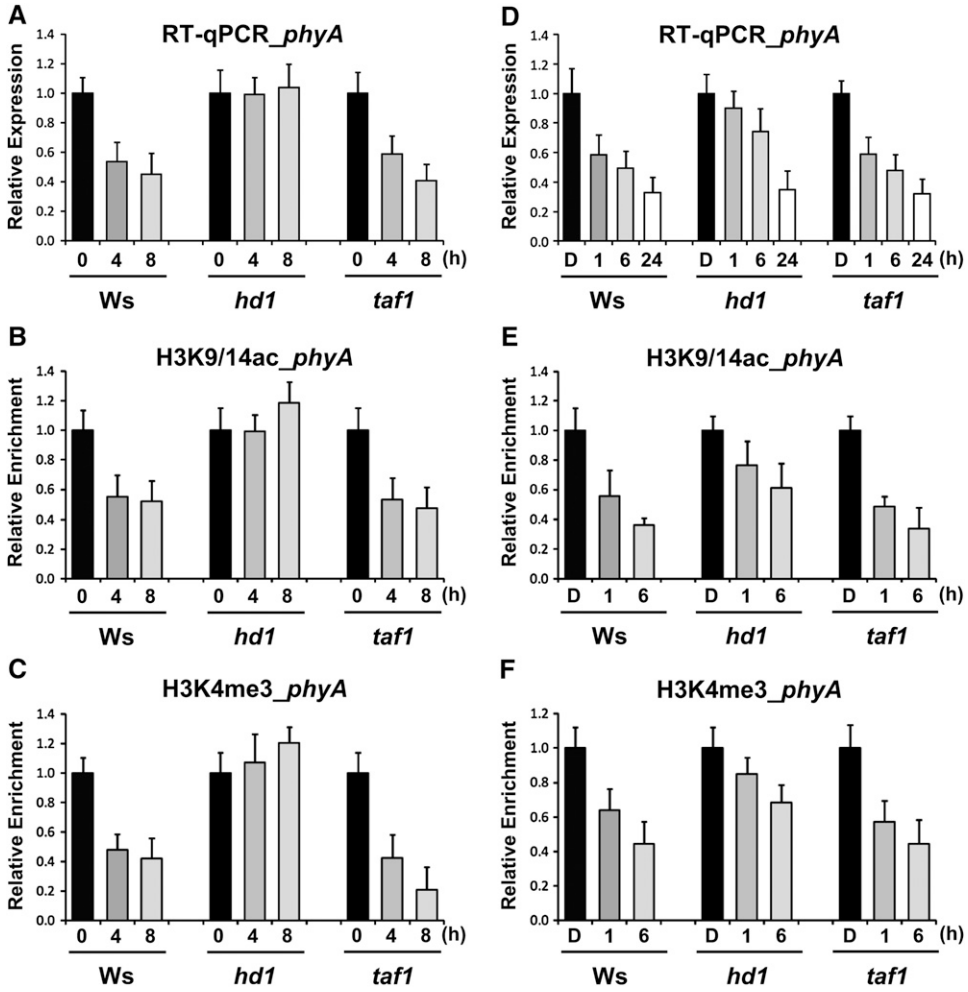


**Figure 6.** Changes of Histone Modifications on *phyA* in *hda19/hd1* and *taf1* Compared with the Wild Type (Ws).

**(A)** Relative *phyA* expression level in Ws, *hda19/hd1*, and *taf1* under the D/L transition. Two-week-old *Arabidopsis* plants were grown under short-day conditions (8 h of L/16 h of D), harvested at the end of the D period (0), and sampled at 4-h intervals (4 and 8) under L conditions. The expression level of *phyA* was determined by quantitative RT-PCR.

**(B)** and **(C)** Relative levels of histone H3 modifications, K9/14ac **(B)** and K4me3 **(C)**, of *phyA* (region 3) in Ws, *hda19/hd1*, and *taf1* under the D/L transition.

**(D)** Relative *phyA* expression level in Ws, *hda19/hd1*, and *taf1* during deetiolation. Four-day-old etiolated *Arabidopsis* seedlings (D) were treated with white light as indicated (1, 6, and 24 h). The expression level of *phyA* was determined by quantitative RT-PCR.

**(E)** and **(F)** Relative levels of histone H3 modifications, K9/14ac **(E)** and K4me3 **(F)**, of *phyA* (region 3) in Ws, *hda19/hd1*, and *taf1* during deetiolation. Error bars indicate SD ($n = 3$).

activating marks, there was a general increase of H3K27ac along the entire transcribed region of active *phyA*, with a moderate peak surrounding the transcription and translation start sites. This result suggests that H3K27ac may be important for transcriptional elongation as well as transcriptional initiation.

At this moment, it is not clear whether all four activating histone marks (H3K4me3, H3K9/14ac, and H3K27ac) are needed incrementally for full *phyA* transcription or if they function redundantly in some combinatorial fashion. In mammalian cells, phosphorylation of H3S10 affects acetylation of H3K9 and H3K14 in an opposite manner and inhibiting acetylation at H3K14 blocks trimethylation of H3K4 (Latham and Dent, 2007; Lee et al., 2010). Whether such crosstalk among histone modifications operates at the *phyA* locus is an interesting regulatory issue for future investigation.

Other than H3 modifications, we have also observed changes in H4 acetylation at K5, K8, K12, and K16, which were rather modest (1.5- to 2.0-fold) compared with those obtained with H3 modifications. Further experiments are needed to assess the contributions of these H4 modifications to *phyA* regulation by D/L.

### Repressive Histone Marks

A simple model for reversible D/L regulation of *phyA* chromatin would be to assume erasure of activating histone marks along

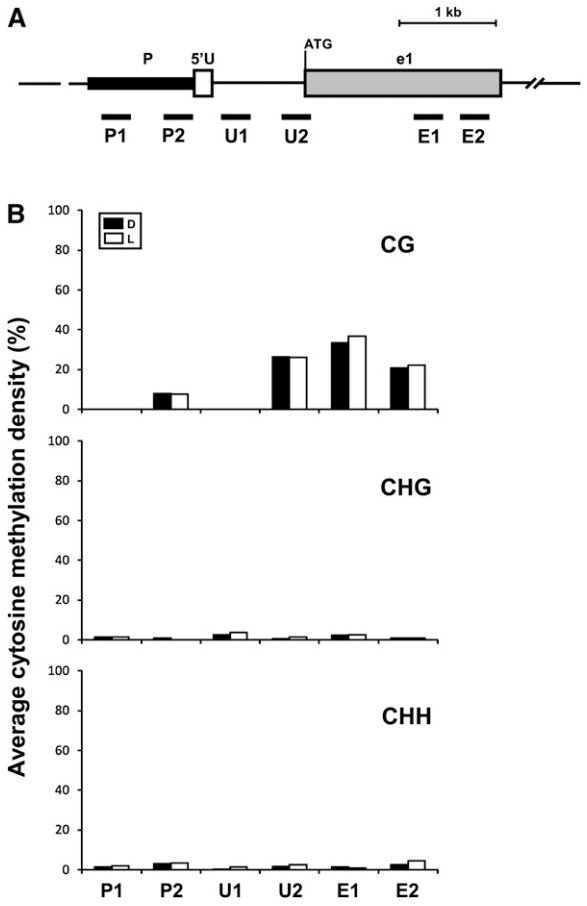


**Figure 7.** Comparison of DNA Methylation Status of the *phyA* Locus in the D/L Transition.

**(A)** Schematic diagram of partial *phyA* gene structure showing the promoter (P), 5′ UTR (5′U), and exon 1 (e1). Black bars below the map represent regions analyzed by bisulfite sequencing. Each fragment was amplified using bisulfite-modified genomic DNA and inserted into pCR2.1-TOPO vector. P1 and P1, Promoter regions; U1 and U2, 5′ UTRs; E1 and E2, exon 1 regions.

**(B)** Analysis of average cytosine methylation (CG, CHG, and CHH) in the D/L transition. Sequences of 10 to 25 clones for each region were analyzed, and the results are shown in Supplemental Figure 9 online. H = A, T, or C.

with L-mediated inactivation of *phyA*. Although deacetylation of H3K9/14 and H3K27 was indeed seen, we also found that *phyA* repression was an active process reinforced by a big increase (12-fold) of the H3K27me3 repressive mark. Genome-wide analyses of *Arabidopsis* have established the importance of H3K27me3 as a major mechanism for silencing of a large number of genes (Turck et al., 2007; Oh et al., 2008; Zhang et al., 2008, Charron et al., 2009). With respect to L-responsive genes, Charron et al. (2009) noted that photosynthetic genes regulated by HY5 and HYH are targeted by acetylation of H3K9 and H3K27 but not by H3K9me3 or H3K27me3. On the other hand, they also found that two genes involved in GA metabolism, GA2ox7 and GA3ox2, that are induced during deetiolation displayed reduced H3K27me3 marks in L. These results suggest the importance of H3K27me3 in restraining GA2ox7 and GA3ox2 expression in D, similar to its role in silencing *phyA* expression in L.

It is significant that the decrease in H3K9ac was not accompanied by an increase in the repressive H3K9me3 mark known to be associated with 40% of *Arabidopsis* genes (Turck et al., 2007). This is perhaps not surprising, as H3K9me3 is associated mostly with heterochromatin, whose silencing depends on small RNA pathways and associated DNA methylation. Here, we present clear evidence that neither small RNA pathways nor DNA methylation is involved in *phyA* repression.

The presence of both activating and repressive histone marks on *phyA* suggests a mechanism to ensure rapid turnoff in the L during deetiolation and transcriptional reactivation when plants are returned to D.

### Relationship to Histone-Modifying Enzymes

The importance of H3K4me3, H3K9/14ac, H3K27ac, and H3K27me3 in D/L regulation of the *phyA* locus implicates enzymes that catalyze reversible acetylation/deacetylation and methylation/demethylation in this process. One working hypothesis is to assume that in D-appropriate histone acetyltransferases, methyltransferases, and demethylases are recruited to the *phyA* locus to demethylate H3K27me3, to acetylate H3K9/14 and H3K27, and to methylate H3K4. In L, similar enzymes are recruited to deacetylate H3K9/14ac and H3K27ac, demethylate H3K4me3, and methylate H3K27.

Since chromatin modifications associated with *phyA* repression are blocked in the *phyB-9* mutant in red light, these changes must be mediated by the phyB signaling pathway, although the precise mechanism is unknown at present.

The *Arabidopsis* genome encodes a large number of histone acetyltransferases/deacetylases and methyltransferases/demethylases; however, the functions of individual enzymes might be hard to determine because of possible functional redundancy. Nevertheless, among the acetylation/deacetylation enzymes, the roles of GCN5, TAF1/HAF2, and HDA19/HD1 in L response have been shown by genetic studies (Bertrand et al., 2005; Benhamed et al., 2006). Mutants deficient in GCN5 and TAF1 are hyposensitive to L with respect to hypocotyl elongation, whereas the reverse is true of the *hda19/hd1* mutant. A simple and straightforward interpretation of these results is that GCN5 and TAF1 are positive regulators whereas HDA19/HD1 is a negative regulator of L responses. For example, GCN5 and/or TAF1/HAF2 might acetylate H3K9/14 in D to activate *phyA* transcription, whereas HDA19/HD1 might deacetylate H3K9/l4ac in L to repress *phyA* expression. Indeed, we found that *hda19/hd1* is unable to down-regulate *phyA* expression in L. Consistent with the higher *phyA* expression in *hda19/hd1* in L, mutant seedlings display L hypersensitivity compared with wild-type seedlings (Benhamed et al., 2006).

Our analysis of histone modifications in *hda19/hd1* uncovered coordinated behavior between the two activating marks, H3K9/14ac and H3K4me3. The mutant *hda19/hd1* is impaired in deacetylation of H3K9/14 in L, which can be attributed to a deficiency of HISTONE DEACETYLASE19. However, to our surprise, this mutant is also unable to demethylate H3K4me3, whose

levels in L are about the same as those in D. This result suggests that deacetylation of H3K9/14ac is required for H3K4me3 demethylation. Whether demethylation of H3K4me3 is also needed for deacetylation of H3K9/14ac remains to be investigated with an appropriate mutant. Crosstalk between H3K4me3 and H3K9/14ac as well as between other histone marks has been reported previously in yeast and human cells (Latham and Dent, 2007; Lee et al., 2010).

Recent evidence shows that in *Arabidopsis*, the chromodomain of LHP1 binds to H3K27me3 of target genes to repress transcription (Turck et al., 2007; Exner et al., 2009). Since the *phyA* locus in L is enriched in H3K27me3, according to this scenario, its reactivation in D during D/L transitions must entail the removal of the effector LHP1 to unmask H3K27me3 so as to enable the demethylation of the latter followed by subsequent acetylation of H3K27. If this model is correct, we would expect *lhp1* mutants to have less efficient repression and therefore display higher *phyA* expression levels in L compared with the wild type. Elucidating the possible role of LHP1 and other histone-modifying enzymes in *phyA* expression will lead to a better understanding of how changes in chromatin structure modify gene expression in response to D/L.

## METHODS

### Plant Materials and Growth Conditions

We used *Arabidopsis thaliana* wild type (Col-0 and Ws), *phyB-9*, *hd1*, *taf1*, *rdr2*, *dcl3-1*, *ddc*, *nrpd1a-2*, and *nrpd1b-1* as plant materials (Reed et al., 1993; Xie et al., 2004; Pontier et al., 2005; Benhamed et al., 2006; Chan et al., 2006). All mutants were in the Col-0 background except *hd1* and *taf1* in the Ws background. Sterilized seeds were incubated on Murashige and Skoog medium supplemented with 1% Suc at 4°C for 4 d and transferred to long-day, short-day, or continuous D conditions. Seedlings were grown for 14 to 21 d in a growth chamber under long-day (16 h of L/8 h of D) or short-day (8 h of L/16 h of D) conditions from cool-white fluorescent light (100 $\mu$mol/m$^2$/s). For deetiolation, sterilized seeds were germinated on Murashige and Skoog medium for 4 d in D after incubation for 4 d at 4°C and then transferred to continuous white light (100 $\mu$mol/m$^2$/s), FR (5 $\mu$mol/m$^2$/s), or R (20 $\mu$mol/m$^2$/s) for the time periods described in the figure legends.

### RNA Extraction and Quantitative RT-PCR

Total RNA isolated from *Arabidopsis* seedlings using Qiagen RNeasy plant mini kits were treated with RNase-free DNase (Qiagen) on column. After quantification of RNA, 1 $\mu$g of total RNA was used for cDNA synthesis using an oligo(dT) and SuperScript III RT kit (Invitrogen). The cDNA was quantified using SYBR Premix Ex Taq (TaKaRa) with gene-specific primers in a Bio-Rad CFX96 real-time system. *ACTIN2* was used as an internal control. The primers used for real-time PCR are listed in Supplemental Table 1 online. Real-time quantitative PCR was repeated with two to five biological replicates, and each sample was assayed in triplicate by PCR. Error bars in each graph indicate SD of three technical repetitions. *ACTIN2* was used as an internal control for normalization in each quantitative PCR experiment.

### ChIP Assay

About 3 g of seedlings was used for ChIP assay. Chromatin preparation and immunoprecipitation were performed as described (Gendrel et al., 2005). Harvested seedlings were fixed in 1% formaldehyde for 10 min in a vacuum. Gly was added to a final concentration of 0.125 M, and the reaction was terminated by incubation for 5 min in a vacuum. Seedlings were rinsed three times with distilled water and frozen in liquid nitrogen. After isolation, chromatin was sheared to ~500-bp fragments by sonication (Diagenode Bioruptor UCD-200). Immunoprecipitation was performed by adding specific antibody in an extract and protein A or G agarose/salmon sperm DNA (Millipore). The antibodies used for ChIP assays were anti-H3K4me2 (Millipore; 07-030), anti-H3K4me3 (Active Motif; 39,159), anti-H3K9/14ac (Millipore; 06-599), anti-H3K9ac (Millipore; 07-352), anti-H3K14ac (Millipore; 07-353), anti-H3K9me2 (Active Motif; 39,239), anti-H3K9me3 (Active Motif; 39,161), anti-H3K27ac (Millipore; 07-360), anti-H3K27me3 (Millipore; 07-449), anti-H3K36me3 (Abcam; ab9050), anti-H4K5ac (Millipore; 07-327), anti-H4K8ac (Millipore; 07-328), anti-H4K12ac (Millipore; 07-595), anti-H4K16ac (Millipore; 07-329), and anti-RNA polymerase II (Santa Cruz Biotechnology; sc-33754). After washing, immune complexes were eluted from the protein A or G beads and reverse cross-linked by incubation for at least 6 h at 65°C. Samples were treated with proteinase K for 1 h at 65°C. DNA was extracted in a final volume of 80 $\mu$L using the QIAquick PCR purification kit (Qiagen). Chip assay was repeated with two to five biological replicates. One microliter of DNA was used for each real-time quantitative PCR with SYBR Premix Ex Taq (TaKaRa) in the CFX96 real-time system (Bio-Rad). Each sample was assayed in triplicate by PCR. Error bars in each graph indicate SD of three technical repetitions. We used *ACTIN2* as internal control for H3K4me2, H3K4me3, H3K9/14ac, H3K9ac, H3K14ac, H3K27ac, H3K36me3, H4K5ac, H4K8ac, H4K12ac, H4K16ac, and anti-RNA polymerase II and *Ta3* for H3K9me2, H3K9me3, and H3K27me3. Primers used for ChIP assays are listed in Supplemental Table 2 online.

### Genomic DNA Isolation, Bisulfite Sequencing, and DNA Methylation Analysis

Methylation status of the *phyA* locus in the D/L transition was evaluated by bisulfite sequencing. Two-week-old *Arabidopsis* plants grown under long-day conditions (16 h of L/8 h of D) were used. Plants at the end of a L period were incubated in D for 24 h and then treated with white light (100 $\mu$mol/m$^2$/s) for 24 h. Genomic DNA was isolated from ~1 g of plant materials treated with continuous D or L for 24 h using the DNeasy plant maxi kit (Qiagen) according to the manufacturer's instructions. Two micrograms of genomic DNA was used for bisulfite treatment using the EpiTect bisulfite kit (Qiagen). Bisulfite DNA conversion was performed in a thermocycler according to the manufacturer's protocol. For each PCR, 1 $\mu$L of bisulfite-treated genomic DNA was used with degenerate primers for amplification of the selected regions of the *phyA* locus. PCR products were inserted into pCR2.1-TOPO (Invitrogen) for blue/white selection. Fifteen to 30 white clones were selected for each sample and then sequenced with M13 forward or M13 reverse primer. The sequences were analyzed using the CyMATE program (http://www.gmi.oeaw.ac.at/en/cymate-index). The primers for sequencing of bisulfite-treated DNAs are listed in Supplemental Table 3 online.

### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *phyA* (At1g09570), *phyB* (At2g18790), *CCA1* (At2g46830), *ACT2* (At5g09810), and *Ta3* (At1g37110).

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Immunoblot Analyses Showing Histone H3 Modifications during Deetiolation or the D/L Transition.

**Supplemental Figure 2.** Relative Levels of Histone H3 Modifications (K4me2, K4me3, K9/14ac, K9me2, K9me3, K27ac, K27me3, and K36me3) at Region 11 Shown in Figure 1B in the D/L Transition.

**Supplemental Figure 3.** Changes of Histone Modifications at Regions 7 (B) and 11 (C) of the *phyA* Locus during Deetiolation.

**Supplemental Figure 4.** Changes of Histone Modifications at Regions 7 (A) and 11 (B) of the *phyA* Locus during the Diurnal Cycle.

**Supplemental Figure 5.** Enrichment of H3K4me3, H3K9/14ac, and H3K27ac at Regions 7 (A) and 11 (B) of the *phyA* Locus in the *phyB* Mutant Exposed to R.

**Supplemental Figure 6.** Changes of Histone Modifications at Regions 7 (A) and 11 (B) of the *phyA* Locus in *hda19*/*hd1* and *taf1* Compared with the Wild Type (Ws).

**Supplemental Figure 7.** *phyA* Expression Levels in RNA–Directed DNA Methylation RdDM-Related Mutants.

**Supplemental Figure 8.** Cytosine Methylation in the Promoter, 5′ UTR, and Exonic Region of *phyA* in the D/L Transition.

**Supplemental Figure 9.** DNA Methylation Analyses of the *phyA* Locus in the D/L Transition Using Bisulfite Sequencing.

**Supplemental Table 1.** Primers for Real-Time Quantitative PCR.

**Supplemental Table 2.** Primers for ChIP Assays.

**Supplemental Table 3.** Degenerate Primers for Bisulfite Sequencing.

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