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Arabidopsis histone deacetylase HDA9 regulates flowering time through repression of AGL19

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ABSTRACT

Flowering time is tightly controlled by several regulatory pathways including photoperiod, vernalization in which epigenetic processes are involved. In this work, we have found that the Arabidopsis histone deacetylase gene *HDA9* is involved in flowering time control. Mutation of the gene led to an early flowering phenotype in short day grown plants while without effect in long days. Analysis of flowering time regulatory gene expression revealed that hda9 mutations highly induced the expression of *AGL19*, but had no effect on *CO*, *SOC1* or *FLC*. Chromatin immunoprecipitation assays indicated that the mutations led to a clear increase of histone H3K9 and H3K27 acetylation on the *AGL19* gene in short days. AGL19 promotes flowering in a way independent of the *CO* and *FLC* pathways and has been shown to be repressed by polycomb group repressive complex2 (PRC2) EMF2 but activated by vernalization. The induced levels of *AGL19* expression and histone acetylation by the *hda9* mutations were comparable to that of the gene under long day conditions, indicating that *AGL19* is regulated also by day length and that HDA9 is involved in short day repression of *AGL19* by promoting histone H3 deacetylation, which may be related to the PRC2 EMF2 complex.

1. Introduction

Chromatin structure and remodeling are basic components of genetic and epigenetic regulations of gene expression in eukaryotes. Chromatin modification and remodeling are regulated by an array of proteins or protein complexes, leading to specific profiles of epigenomes which include DNA methylation, covalent modifications of the N-terminal tails of the core histones, histone variant deposition, nucleosome positioning and compaction and chromatin protein association. Specific combinations of histone modifications have been identified to be associated with active or repressed states of the cognate chromatin [1,2]. Histone modifications including acetylation, methylation and ubiquitination have been found to be involved in the regulation of gene expression related to many developmental and stress responsive pathways in plants [3–5].

Flowering transition in Arabidopsis is one of the plant developmental pathways that have been demonstrated to be particularly regulated by chromatin-based epigenetic mechanisms [6]. Flowering time in Arabidopsis is sensitive to photoperiod and vernalisation and is also regulated by the autonomous pathway. Photoperiod pathway involves induction of the key transcription factor CONSTANS (CO) that activates downstream flowering promoting regulators [7]. Molecular mechanism of vernalization has been studied extensively in Arabidopsis, which acts mainly at the epigenetic level to stably repress *FLC* expression [8]. Vernalization induces the expression of the PHD-domain protein VIN3 [9]. VIN3 protein is thought to integrate cold signal to induce chromatin modifications at the *FLC* locus, mainly histone H3 lysine 27 trimethylation (H3K27me3) by polycomb group repressive complexe2 (PRC2) containing VRN2, CLF/SWN, FIE, and possibly VIN3 [8]. This effect overrides other types of regulation such as by the autonomous pathway. Besides repression of *FLC* and *FLC*-related genes, vernalization – activated *VIN3* is also involved in the activation of flowering promoting genes such as *AGL24* and *AGL19* [10–12]. *AGL24* and *AGL19* are MADS box genes and function as activators of flowering in response to vernalization. Ectopic expression of the genes can induce flowering [10,11]. Chromatin immunoprecipitation assays demonstrated that *AGL19* chromatin is enriched in repressive H3K27me3 before, but much less after, vernalization [11]. H3K27me3 at *AGL19* is most likely deposited by a PRC2 complex composed of EMF2, CLF/SWN, FIE, and MSI1 [11], different from that for H3K27me3 at *FLC* [13]. However, the precise mechanism of *AGL19* repression and VIN3-dependent activation of the gene is not clear.

Histone acetylation homeostasis is regulated by antagonistic actions of histone acetyltransferases (HATs) and histone deacetylases (HDAC). Plant genome contains about 20 HDAC genes belonging to three classes. Among them, two have primary homology to

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yeast HDAC groups: RPD3 (Reduce Potassium Dependency 3), and SIR2 (Silent Information Regulation2). The third group known as the HD2 class is only found in plants. During the last years, the biochemical or developmental function of a number of HAT and HDAC has been characterized in plants [1–3]. However, the function of most of HDAC genes in Arabidopsis is unknown. In this work we characterized T-DNA insertion mutations of 3 HDAC genes. We found that loss-of-function of *HDA9* induced an early flowering phenotype under short day conditions. Further analysis revealed that the *hda19* mutations induced *AGL19* expression and histone H3 acetylation on the gene under short days. The data indicate that HDA9 represses flowering by repressing *AGL19* gene expression independently of CO or FLC pathways.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana lines in this study were in the Col-0 or Ws accessions. The primers used for genotyping are indicated in Supplemental Table 1. Arabidopsis seeds were sowed *in vitro* on 0.5× Murashige Skoog (MS) media, incubated at 4 °C for 48 h, transferred to a growth chamber (20 °C) under white light (120 μmol m^{-2} s^{-1}) in LD (16 h light/day) or SD (8 h light/day) photoperiods. For measuring flowering time, 10-day-old *in vitro* seedlings were transferred onto soil and kept in growth chambers under LD or SD photoperiods. The flowering time was measured as the number of total rosette leaves longer than 0.5 cm at bolting for at least 15 plants. Graphs show means ± standard deviation.

2.2. Expression analysis

Total RNA was isolated from 12-day-old *in vitro* seedlings from wild type, *hda9-1* and *hda9-2* mutants using Nucleospin RNA plant kit (Macherey–Nagel) according to manufacturer's instructions. cDNA was synthesized from 3 μg of RNA by ImProm-II™ Reverse Transcriptase (Promega) and analyzed by real-time PCR. For real-time PCR, the reactions were performed in a 96-well plate with a Light Cycler 480 system (Roche), using SYBR Green to monitor dsDNA synthesis. Reactions contained 10 μl 2× SYBR Green Master Mix reagent (Roche), 200 nM of each gene-specific primer and template DNA in a final volume of 20 μl. The following standard thermal profile was used for all PCR reactions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplicon dissociation curves, i.e., melting curves, were recorded after 40 cycles by heating from 60 °C to 95 °C with a ramp speed of 1.9 °C/min. Data were analyzed using the Light Cycler 480 software 1.5.0 (Roche). Primers used are listed in Supplemental Table 1.

2.3. Western blot assays

The protein extracts were resolved on SDS–PAGE and blotted to a nylon membrane and followed by incubation with anti-acetyl-histone H3 (Millipore, catalog No. 05-599), anti-acetyl-histone H4 (Millipore, catalog No. 06-598) and anti-histone H3 (Millipore, catalog No. 05-499). The immunoreactions were revealed by enhanced chemiluminescence.

2.4. Chromatin immunoprecipitation assays

Seedlings grown in SD or LD conditions were harvested and fixed in 1% formaldehyde for 15 min in a vacuum and then neutralized by 0.125 M Gly. After washing with sterilized water, the samples were ground in liquid nitrogen. Nuclei pellets were suspended in a buffer containing 0.25 M sucrose, 10 mM Tris–HCl, pH 8, 10 mM $MgCl_2$, 1% Triton X-100, 5 mMβ-mercaptoethanol, 0.1 mM PMSF, and protease inhibitors (one minitablet per milliliter; Roche). The suspensions were transferred to microfuge tubes and centrifuged at 12,000 g for 10 min. The pellets were suspended in 1.7 M sucrose, 10 mM Tris–HCl, pH 8, 2 mM $MgCl_2$, 0.15% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF, and protease inhibitors and centrifuged through a layer of the same buffer in microfuge tubes. The nuclear pellets were suspended in a buffer containing 50 mM Tris–HCl, pH 8, 10 mM EDTA, 1% SDS, and protease inhibitors. The nuclei were sonicated 4 times for 15 s at 4 °C followed by centrifugation. The supernatants containing chromatin fragments were diluted 10-fold with 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8, and 167 mM NaCl. A 1 mL aliquot of the dilution was used for an immunoprecipitation assay using anti-acetyl-histone H3 lysine 9 (Millipore, catalog No. 07-352) and anti-acetyl-histone H3 lysine 27 (Millipore, catalog No. 07-360) (5 μg of antibody for 1 mL of diluted protein lysate) antibodies. Three fully independent ChIP assays using samples collected separately were performed and qPCR analyses were performed at least in duplicate on each biological replicate using specific primers (Supplemental Table 1).

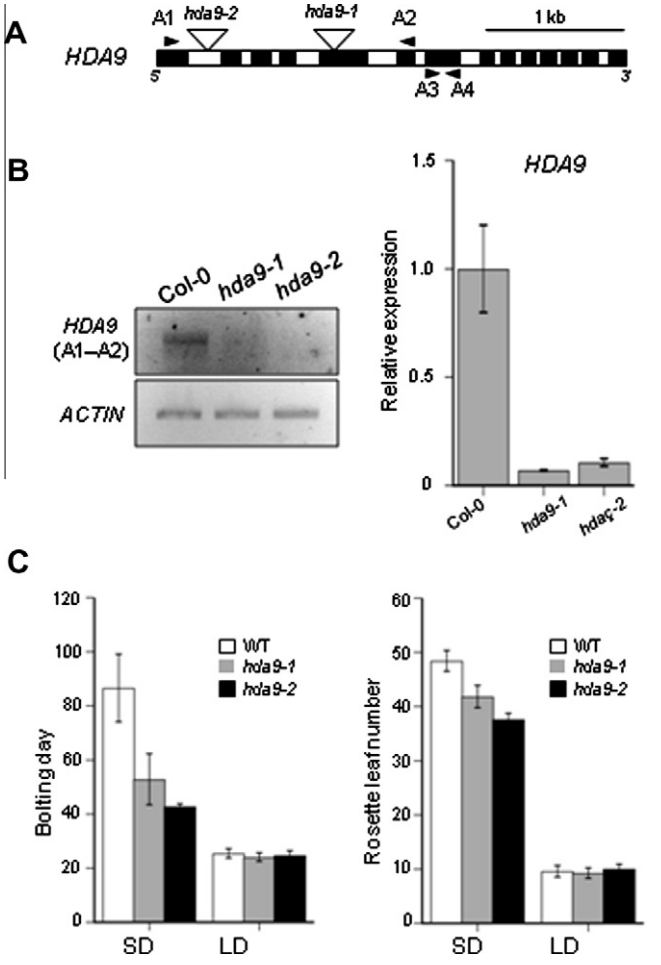

Fig. 1. Loss-of-function mutations of *HDA9* lead to early flowering in short days. A. HDA9 gene structure. Positions of T-DNA insertions of *hda9-1* and *hda9-2* alleles are indicated by open triangles. Primers used for expression analysis are indicated by arrow heads. B. *HDA9* transcripts are not accumulated in the insertion mutants analyzed by RT-PCR with primers A1 and A2 (left) and by quantitative RT-PCR with primers A3 and A4 (right). C. Flowering time of wild type and *hda9-1* and *hda9-2* plant under short day and long day conditions. Bolting days and leaf numbers at bolting day were surveyed. The vertical T-bars indicate standard deviation from means of 20 plants.

3. Results

3.1. Analysis of Arabidopsis HDAC gene mutants reveals that HDA9 is involved in flowering time control in short days

The function of most HDAC genes in Arabidopsis was not known. To study the function of Arabidopsis HDAC genes, we characterized T-DNA mutants of Rpd3 family members HDA9, HDA15 and HDA18. RPD3 genes can be divided into 3 subclasses. HDA9 belongs to subclass I, HDA15 and HDA18 to subclass II. The mutants did not show any visible morphological phenotype under normal long day (16 h/8 h) growth conditions, except the *hda9-1* and *hda9-2* mutants displayed an early flowering phenotype under short day conditions, with 30 days less (or 8–10 rosette leaves less) in average compared to wild type (Fig. 1), indicating that wild type HDA9 may repress flowering in short days. Double mutants were obtained by crosses between *hda9* and *hda15*. The double mutants did not show any phenotype and did not enhance the early flowering phenotype (Fig. S1), suggesting the HDA9 function in flowering time control may be independent of *HDA15*.

3.2. HDA9 represses AGL19 expression under short day

The above data suggested that HDA9 may be involved in the repression of flowering promoting genes under short day condition. Short day condition is repressive for flowering in Arabidopsis under which the long day photoperiod signal integrator gene *CO* is repressed. We therefore first checked whether the *had9* mutations altered the expression of *CO*. Wild type and mutant seedlings at 11–12 days were collected every 4 h during a period of 36 h and analyzed for *CO* expression by realtime RT-PCR. No clear difference in *CO* expression was observed between wild type and the *hda9* mutant alleles (Fig. S2). In addition, the expression of *SOC1*, a downstream target gene of *CO* was not clearly affected either by the mutations under long day or short day conditions (Fig. 2). These data suggested that HDA9 function in flowering time control may be independent of the CO pathway. Furthermore, the mRNA levels of *FLC* were not altered by the *hda9* mutations (Fig. 2). By contrast, *AGL19* mRNA levels in the mutants were several folds higher than in wild type under short days, whereas no clear difference between wild type and the mutants was observed under long days (Fig. 2). In addition, AGL19 mRNA level was much higher in long day than short day conditions, which suggests that in addition to vernalization, *AGL19* is also activated by long day conditions, corroborating the role of AGL19 in flowering activation.

3.3. HDA9 regulates histone H3 acetylation over the AGL19 promoter

To study whether the mutations of *HDA9* affected histone modification, histones isolated from wild type, *hda9-1* and *hda9-2* seedlings were analyzed by Western blot using antibodies of acetylated histone H3, acetylated histone H4 and total histone H3. The analysis revealed an increase of acetylated H3 in the two mutant lines, whereas the levels of acetylated H4 and total H3 were about the same between wild type and the mutants (Fig. 3). These data suggested that HDA9 may be mainly involved in deacetylation of histone H3.

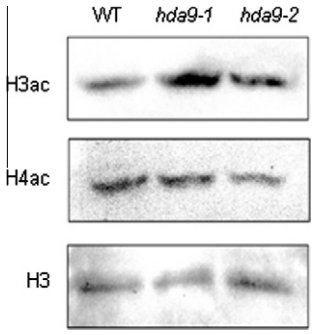

Fig. 3. The mutations of *HDA9* augmented acetylated histone H3. Histone proteins isolated from wild type and the *hda9* mutant plants were analyzed by Western blots using antibodies against histone H3, acetylated H3 and acetylated H4 as indicated on the left.

To study whether the mutation affected histone acetylation over flowering regulatory genes, we performed chromatin immunoprecipitation analysis of wild type and mutant seedlings grown under long day and short day conditions, by using antibodies of H3K9ac and H3K27ac. Because acetylation is most enriched in the promoter regions, we analyzed the 5′ end of *AGL19*, *FLC* and *SOC1*. In the wild type, the acetylation levels at H3K9 and H3K27 on *AGL19* were much lower (5–30 times) under short day than under long day conditions, correlating with the expression levels of the genes in both conditions (Fig. 4). In the *hda9* mutants grown under short day conditions, H3K9 and H3K27 acetylation levels on the gene were increased to that of wild type under long day conditions (Fig. 4). These observations indicate that HDA9 suppresses histone H3 acetylation on *AGL19* under short day condition. For *FLC* and *SOC1*, there was no clear variation of H3K9 and H3K27 acetylation between wild type and mutants and between long and short days (Fig. 4).

4. Discussion

4.1. HDA9 is a negative regulator of flowering

Many chromatin regulators have a function in flowering time control, most of which are involved in the expression of *FLC* [4,6]. In this work we have shown that HDA9 is involved in the

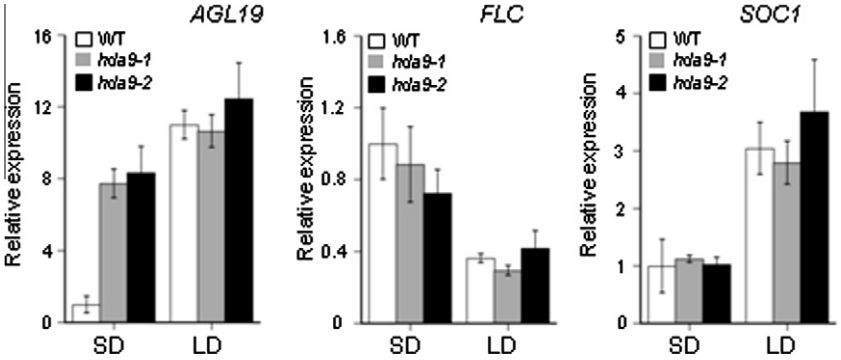

Fig. 2. *HDA9* mutations lead to derepression of *AGL19* in short days. mRNA levels of *AGL19*, *FLC* and *SOC1* in wild type and the *hda9* mutants grown under long day and short day conditions were analyzed by quantitative RT-PCR. Transcript levels of each gene in wild type under short days are assessed arbitrarily at 1.

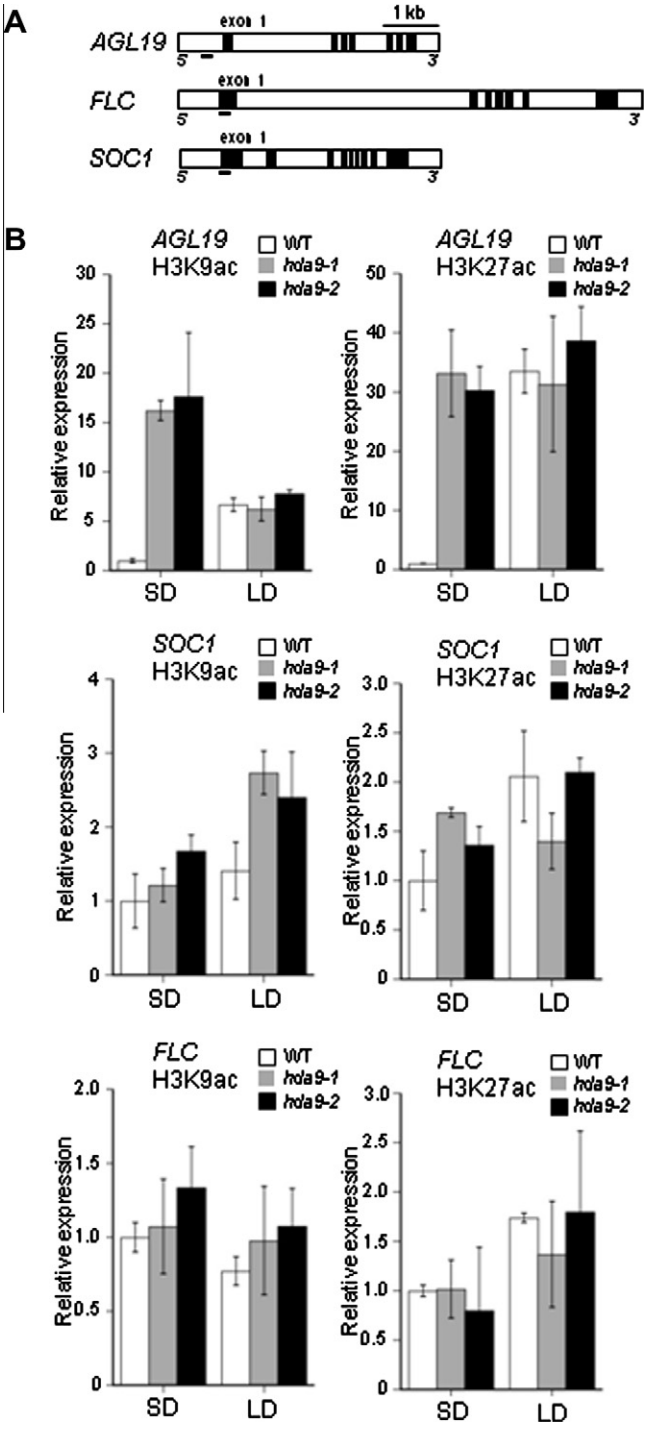

Fig. 4. The mutations of *HDA9* lead to increased acetylation of histone H3K9 and H3K27 on *AGL19* promoter under short days. Chromatins isolated from wild type and the *hda9* mutants grown under long and short days were immune-precipitated by anti H3K9ac and H3K27ac and analyzed by PCR using primer set corresponding to the promoter regions of *AGL19*, *SOC1* and *FLC*, as indicated. The region at the 5′ end of the genes tested for chromatin immunoprecipitation are underlined in A.

regulation of *AGL19* that acts in a pathway independent of *FLC* and *SOC1* to promote flowering. *AGL19* is shown to be activated by vernalization through an undefined mechanism [11]. The present data showing that *AGL19* expression was repressed under short day, but activated under long day, suggesting that *AGL19* is not just subjected to vernalization regulation, but is also activated under other flowering-favorable conditions such as long day photoperiods in Arabidopsis. Likely, *AGL19* functions as a general flowering promoting regulator, as its over-expression could stimulate flowering in both long day and short day conditions [9,10]. The finding that the repression of *AGL19* was correlated with low level of histone H3 acetylation in short day and that the mutation of *HDA9* led to increased H3 acetylation and derepression of *AGL19* in short day allows considering HDA9 as a general negative regulator of flowering by repressing *AGL19* expression in Arabidopsis.

AGL19 is shown to be subjected to epigenetic regulation [11]. Chromatin immunoprecipitation assays demonstrated that *AGL19* chromatin is enriched in repressive H3K27me3 before, but much less after, vernalization [11]. Because histone methylation and acetylation are mutually exclusive from the same residues, high H3K27me3 correlates with lower H3K27ac on *AGL19* in repressed state (Fig. 4). Likewise, histone H3 (especially H3K27) deacetylation may be required for subsequent H3K27 trimethylation to establish or to mark the repressive state of *AGL19*. H3K27me3 at *AGL19* is most likely to be deposited by the PRC2 EMF2 complex [11]. Generally, PRC2 complexes are found to be associated with a HDAC in other eukaryotes, such an HDAC has not been identified in plants. It would be interesting to known whether HDA9 makes part of the EMF2 complex in Arabidopsis.

4.2. Arabidopsis HDAC genes function

The Western blot data showing increases of overall H3 acetylation in *hda9* mutants suggest that HDA9 may be also involved in the repression of many other genes, despite that there is no morphological phenotype observed in the mutants. Similarly, T-DNA insertion mutants of 2 additional HDAC genes did not produce any visible phenotype under normal growth conditions. In fact, except HDA19 (also called HD1), many of loss-of-function mutants of Arabidopsis HDAC genes display no morphological phenotype, but exhibit defects or abnormality in stress or hormonal responses [3,5]. HDA19 is thought to be the primary HDAC for site-specific gene regulation, important for developmental key regulatory gene expression in Arabidopsis [3,5]. A similar situation was found in rice HDAC genes [14]. It is attempting to postulate that histone acetylation regulated by these HDACs may be mostly involved in inducible gene expression or in adjusting the levels of gene expression in Arabidopsis. The fact that the double mutants of HDAC genes produced in this study did not aggravate the phenotype is in favor of the above hypothesis and also suggests that these HDAC may have different targets or function at a different timing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.102.

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