PICKLE associates with histone deacetylase 9 to mediate vegetative phase change in Arabidopsis

Tieqiang Hu, Darren Manuela, Valerie Hinsch and Mingli Xu

Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA

Summary

- The juvenile-to-adult vegetative phase change in flowering plants is mediated by a decrease in miR156 levels. Downregulation of MIR156A/MIR156C, the two major sources of miR156, is accompanied by a decrease in acetylation of histone 3 lysine 27 (H3K27ac) and an increase in trimethylation of H3K27 (H3K27me3) at MIR156A/MIR156C in Arabidopsis.
- Here, we show that histone deacetylase 9 (HDA9) is recruited to MIR156A/MIR156C during the juvenile phase and associates with the CHD3 chromatin remodeler PICKLE (PKL) to erase H3K27ac at MIR156A/MIR156C.
- H2Aub and H3K27me3 become enriched at MIR156A/MIR156C, and the recruitment of Polycomb Repressive Complex 2 (PRC2) to MIR156A/MIR156C is partially dependent on the activities of PKL and HDA9.
- Our results suggest that PKL associates with histone deacetylases to erase H3K27ac and promote PRC1 and PRC2 activities to mediate vegetative phase change and maintain plants in the adult phase after the phase transition.

Introduction

During the vegetative development of flowering plants, the shoot produces morphologically distinct leaves (juvenile and adult leaves) before flowering (Poethig, 2003, 2013). The juvenile-to-adult phase transition (vegetative phase change) is largely controlled by the conserved miR156-SPL module (Willmann & Poethig, 2007; Wu et al., 2009; Wang et al., 2011; Leichty & Poethig, 2019), which also regulates floral induction, the immune response, herbivore resistance, and grain development (Jiao et al., 2010; Mao et al., 2017; Wang et al., 2018; Hyun et al., 2019). Transcription of MIR156A/MIR156C, the two major sources of mature miR156 in Arabidopsis, is controlled by epigenetic factors (Pícó et al., 2015; Xu et al., 2016; Y. Xu et al., 2016; Xu et al., 2018; Fouracre et al., 2021), components of the Mediator complex such as CENTER CITY (CCT)/MEDIATOR12 (MED12) and GRAND CENTRAL (GCT)/MED13 (Gillmor et al., 2014; Buendia-Monreal & Gillmor, 2017), carbohydrates (L. Yang et al., 2013; Yu et al., 2013; Lawrence et al., 2020; Xu et al., 2021) and hormones such as ABA and hormone-related factors (Guo et al., 2017, 2021; Tian et al., 2020).

Among the epigenetic factors involved in transcriptional regulation of MIR156A/MIR156C are the CHD3 chromatin remodeler, PICKLE (PKL) and the SWI2/SNF2 ATPase, BRAHMA (BRM). PICKLE promotes, while BRM prevents, the formation of nucleosomes near the transcription start site (TSS) of MIR156A/MIR156C (Xu et al., 2016a; Y. Xu et al., 2016). In addition to modulating chromatin structure, PKL also promotes trimethylation of lysine 27 on H3 (H3K27me3) at a variety of targets (Zhang et al., 2008, 2012; Xu et al., 2016a), while BRM has been reported to prevent H3K27me3 deposition at various targets (Li et al., 2015; Y. Xu et al., 2016). H3K27me3 is catalysed by Polycomb Repressive Complex 2 (PRC2), which is generally associated with transcriptionally repressed genes (Zhang et al., 2007a; Lafos et al., 2011; Li et al., 2015). BRM occupancy prevents the association of PRC2, therefore preventing the deposition of H3K27me3 (Li et al., 2015). It is however unknown how PKL promotes H3K27me3 deposition.

Most of the genes encoding PRC2 components are not expressed in a temporally changing pattern during vegetative development. However, the FERTILIZATION INDEPENDENT ENDOSPERM (FIE) protein, which is a component of all PRC2 complexes in Arabidopsis, is temporally associated with MIR156A/MIR156C chromatin, indicating that PRC2 is temporally recruited to these genes (Xu et al., 2016a). H3K27me3 may be promoted by decreasing H3K4me3 levels, as H3K4me3 levels at MIR156A/MIR156C are inversely correlated with H3K27me3 levels. H3K4me3 is catalysed by the SET domain protein ARABIDOPSIS TRITHORAX RELATED 7 (ATXR7) and promoted by the SWR1 complex whose activity promotes the exchange of histone variant H2A.Z for H2A (Tamada et al., 2009; Choi et al., 2013; Xu et al., 2018). Mutation in the SWR1 complex resulted in decreased levels of H3K4me3 at both MIR156A and MIR156C, and increased levels of H3K27me3 at MIR156A but not at MIR156C. This suggests that reducing H3K4me3 levels is not sufficient to promote H3K27me3. PRC1 and PRC2 are co-localised at transcriptionally repressed genes, and the B3 transcription factor VIVIPAROUS/ABI3-LIKE (VAL1) and VAL2 can recruit PRC1 to these loci (Turck et al., 2021).
During epigenetic silencing of FLOWERING LOCUS C (FLC), VAL1 triggers the deposition of H3K27me3 at FLC and consequently repression of FLC (Qiuesta et al., 2016). VAL1 and VAL2 and the PRC1 RING finger protein Arabidopsis thaliana B Lymphoma MO-MLV Insertion Region 1A/1B (AtBMI1A/1B) mediate ubiquitination of H2A (H2Aub) and H3K27me3 at MIR156A/MIR156C (Picó et al., 2015; Fourcade et al., 2021). However, VAL1/2 regulate the overall levels rather than the temporal pattern of MIR156A/MIR156C expression (Fourcade et al., 2021), leaving the factors that regulate the temporal recruitment of PRC2 to MIR156A/MIR156C to be determined.

Whereas trimethylation of H3K27 represses transcription, acetylation of this residue has the opposite effect. Consistent with this general observation, the level of H3K27ac at MIR156A/MIR156C is high during the juvenile phase, and declines as the shoot ages (Xu et al., 2016a). HISTONE DEACETYLASE 9 (HD9) erases H3K27ac globally (Chen et al., 2016; Kim et al., 2016; Zeng et al., 2020), and the removal of the acetyl group at H3K27 is a prerequisite for trimethylation of H3K27 at FLC (Zeng et al., 2020). HDA9 physically interacts with the SANT (SWI3/DAD2/N-CoR/TFIII-B) domain protein POWERDRESS (PWR) to regulate flowering (Kim et al., 2020) and to promote the onset of age-related and dark-induced leaf senescence (Chen et al., 2016). HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 15 (HOS15) is another component of the HDA9–PWR complex and has similar roles in plant development in addition to immune and stress responses (Mayer et al., 2019; Park et al., 2019; Yang et al., 2020). HDA9 also promotes thermomorphogenesis by assisting in eviction of H2A.Z at the auxin biosynthesis gene YUCCA8 (YUC8), and in the deacetylation of H3K9K14 at the YUC8 locus (van der Woude et al., 2019).

In plants, the CHD3/4 chromatin remodeler PKL has been reported to regulate root cell differentiation, flowering, seedling differentiation, and vegetative phase change through modulating levels of H3K27me3 at targets (Li et al., 2005; Zhang et al., 2008, 2012; Aichinger et al., 2009, 2011; Xu et al., 2016a). PICKLE also has roles in reducing H3K27ac levels at some targets (Zhang et al., 2012; Xu et al., 2016a). However, PKL was reported to primarily exist as a monomer (Ho et al., 2013), and how PKL acts to reduce H3K27ac and increase H3K27me3 has not been determined. Here we show that HDA9 is temporally recruited to MIR156A/MIR156C, and it associates with PKL to decrease H3K27ac levels and increase H2Aub and H3K27me3 levels at MIR156A/MIR156C to mediate vegetative phase change. Our results suggest the presence of a PKL–HDA9 complex and further suggest that this complex promotes the activities of PRC1 and PRC2 at MIR156A/MIR156C chromatin.

Materials and Methods

Plant material and growth conditions

All the Arabidopsis stocks used in this study were on the Col background. pkl–10, sun–3 have been described previously (Xu et al., 2016a). hda6–7 (cs66154) and hda9–1 (SALK_007123) were obtained from the ABRC. Fragments of the FIE promoter and coding region were cloned using the Golden Gate method (Engler et al., 2014) and a 6× HA tag was inserted after the FIE coding region (Wood et al., 2006; Deng et al., 2013). The FIE–HA construct was then transformed into fie–11 heterozygotes, and plant homozygous for fie–11 with a wild-type phenotype was selected for FIE binding assays. Similarly, HDA6–HA, HDA6–GFP, HDA9–HA, HDA9–GFP and PKL–GFP lines were constructed by the Golden Gate system and transformed into hda6–7, hda9–1 or pkl–10 mutants. Lines that have complemented the corresponding hda6–7, hda9–1 and pkl–10 mutant were selected for western blot, co-immunoprecipitation (Co-IP) and chromatin IP (ChIP) analysis. Seeds were sown on Sunshine SS#8F2 potting soil, stratified at 4°C for 2–4 d, and then transferred to Conviron growth chambers maintained at a constant 22°C in either LDs (16 h : 8 h, light : dark) or short-days (SD) (10 h : 14 h, light : dark). Unless otherwise specified, all of the data presented in this article were obtained from plants growing under SDs. Plant age was measured from the date when pots were transferred to growth chambers.

Quantitative RT-PCR

Shoot apices of SD grown plants were harvested and total RNA from them was extracted using TRIzol reagent (Invitrogen), followed by Turbo DNase (Ambion) treatment, according to the manufacturer’s instructions. cDNA was reverse transcribed from 1 μg of RNA using SuperScript III reverse transcriptase (Invitrogen), and qPCR was performed using a Bio-Rad CFX96 real-time system. Primers used for qPCR are listed in Supporting Information Table S1.

Co-immunoprecipitation

HDA6–HA hda6–7 and HDA9–HA hda9–1 were crossed to PKL–GFP pkl–10 and plants homozygotes for each were selected for Co-IP analysis. In total, 1 g of seedlings from each genotype was ground in liquid nitrogen and 2 ml of extraction buffer (50 mM pH 7.5 Tris–HCl, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 5 mM MgCl2, 1.5 mM CaCl2, 1 mM PMSF, 0.5% NP-40 detergent, 10% glycerol, Protease Inhibitor Cocktail Sigma P9599) were added to the fine powder. The crude extract was centrifuged twice at 17 000 g for 10 min. The supernatant after centrifugation was then collected for IP assays. Next, 25 μl GFP–Trap magnetic beads (Chromotek, Islandia, NY, USA) were added to each tube for IP and gently rocked overnight at 4°C. The beads were then washed with washing buffer (50 mM pH 7.5 Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40 detergent, 10% glycerol) three times and eluted with 1× reduced Laemml sample buffer. The eluted substances, as well as inputs, were subjected to western blot analysis. Antibodies against HA (12CA5; Sigma) and GFP (A11122; Invitrogen) were used as the primary antibodies in western blot analysis, followed by HRP-linked anti-rabbit IgG (7074; Cell Signalling, Danvers, MA, USA) or HRP-linked anti-mouse IgG (7076; Cell Signalling) as secondary antibodies. The membrane was incubated using Amersham Western
Chromatin immunoprecipitation

Here, 0.5–1.0 g 1-wk-old, 2-wk-old, or 3-wk-old shoot apices were harvested and fixed with 1% formaldehyde under vacuum for 15 min. Tissues were ground in liquid nitrogen and suspended in extraction buffer 1 (0.4 M sucrose, 10 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, 5 mM β-mercaptoethanol, 1 mM PMSF, and 0.1% Triton X-100). Pellets were washed with extraction buffer 2 (0.25 M sucrose, 10 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, 5 mM β-mercaptoethanol, 1 mM PMSF, and 1% Triton X-100), and resuspended in nuclei lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA and 1% SDS). DNA was then diluted in buffer (1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.0, 167 mM NaCl and 0.01% SDS) and sonicated using a Covaris ultrasonicator M220. Next, 1% of antibodies against HA (12CA5; Sigma), GFP (A11122; Invitrogen), H3 Abcam (ab1791, Waltham, MA, USA), H3K27me3 Millipore (07-449), H3K27ac Abcam (ab4729) or H2Aub (8240S; Cell Signalling) were used in IP. The IP protein and DNA were reverse crosslinked, and DNA was isolated using the QIA Quick PCR purification kit (Qiagen). qPCR was performed on a Bio-Rad CFX96 real-time system after DNA was extracted from the IP product. For H3K27me3 and H3K27ac, STM was used as a control locus (Angel et al., 2016; Fouracre et al., 2011; Coustham et al., 2012; Qieta et al., 2016; Xu et al., 2016a; Fouracre et al., 2021), and data are presented as a ratio of (H3K27me3 gene of interest/H3 gene of interest) to (H3K27me3 STM/H3 STM) or (H3K27ac gene of interest/H3 gene of interest) to (H3K27ac STM/H3 STM). For H2Aub, ABI3 was used as a control locus (C. Yang et al., 2013; Qieta et al., 2016; Fouracre et al., 2021) and data are presented as the ratio of (H2Aub gene of interest/H3 gene of interest) to (H2Aub ABI3/H3 ABI3). For GFP ChIP, TA2 was used as a control locus and data are presented as the ratio of (GFP ChIP gene of interest/GFP input gene of interest) to (wild-type ChIP gene of interest/wild-type input gene of interest) normalised to the TA2 locus. Fold enrichment in the HDA6-HA ChIP analysis was calculated following the same procedure as in the GFP ChIP analysis. Primers for ChIP analysis are listed in Table S1.

Western blot

Nuclear proteins were isolated from 2-wk-old Col, plc-10, bda9-1 and plc-10 bda9-1 using the same extraction buffer 1, extraction buffer 2 and nuclei lysis buffer as used for ChIP analysis. The nuclear proteins were resolved on a 4–12% SDS-PAGE gradient gel and transferred to a membrane. The membrane was incubated with anti-H3 (ab1791), anti-H3K27ac (ab4729), anti-H3K27me3 (07-449; Millipore), and H2Aub (8240S; Cell Signalling) as first antibodies, followed by HRP-linked anti-rabbit IgG (7074; Cell Signalling) as the secondary antibody. The membrane was incubated using Amersham Western Blotting Detection Reagents and detected and analysed on the Amersham ImageQuant 800 system.

Results

HDA6 and HDA9 promote vegetative phase change through miR156-independent and miR156-dependent pathways, respectively

The juvenile-to-adult vegetative phase transition is mediated by the downregulation of pri-miR156a and pri-miR156c, the two major sources of mature miR156 (Wu et al., 2009; Xu et al., 2016a; He et al., 2018). The downregulation of pri-miR156a and pri-miR156c is correlated with a decrease in H3K27ac and an increase in H3K27me3 at MIR156A/MIR156C (Xu et al., 2016a). Previous work has shown that the CHD3 chromatin remodeler PKL promotes a decrease in H3K27ac and an increase in H3K27me3 at these loci (Xu et al., 2016a), but its mechanism remains unknown. HDA6 and HDA9 are reported to remove histone acetylation and to induce downregulation of gene expression in plants (Probst et al., 2004; Earley et al., 2006; Tanaka et al., 2008; van Zanten et al., 2014; Chen et al., 2016; Kim et al., 2016; Mayer et al., 2019; Zeng et al., 2020). To determine if these genes play a role in vegetative phase change, we examined the phenotype of the null alleles hda6-7 (rts1-1) (Aufsatz et al., 2002) and hda9-1 (van Zanten et al., 2014; Chen et al., 2016; Kim et al., 2016). In Arabidopsis, juvenile shoots have a relatively short plastochron and produce leaves that are relatively small and round and lack trichomes on their abaxial surface, whereas adult shoots have a longer plastochron and produce leaves that are larger, more elongated and have trichomes on their abaxial surface (Telfer et al., 1997; Wang et al., 2008; Wu et al., 2009). The rosette of hda6-7 resembled wild-type Col, whereas the rosette of hda9-1 was slightly smaller and had more leaves than Col rosettes of the same age, demonstrating that hda9-1 has a shorter plastochron than Col (Fig. 1a). Under SD conditions, the first leaf of abaxial trichomes in Col was leaf 8.4, and was delayed by c. 2 leaves in hda6-7 and 4 leaves in hda9-1 (Fig. 1b). Abaxial trichome production in hda6-7 hda9-1 was not significantly different from hda9-1 (Fig. 1b). Juvenile leaves are rounder and have a smaller leaf blade length : width ratio than the adult leaves (Xu et al., 2016a,b; He et al., 2018). The leaf blade length : width ratio of the 5th and 7th leaves of hda9-1 was significantly smaller than that of the 5th and 7th leaves of Col, while the leaf blade length : width ratio of the 5th and 7th leaves of hda6-7 was not significantly different from Col; there was no significant difference in the length : width ratio of hda9-1 and hda6-7 hda9-1 (Fig. 1a,c). These results suggested that HDA6 and HDA9 regulated vegetative phase change by different mechanisms.

To investigate if HDA6 and HDA9 regulated vegetative phase change by modulating the amount of miR156, we used RT-qPCR to examine the level of miR156 in the shoot apices of Col, hda6-7, hda9-1, and hda6-7 hda9-1 at 8, 12, 16 and 20 d after planting. hda6-7 had no effect on the level of miR156, but hda9-1 and hda6-7 hda9-1 had elevated levels of miR156 at each of these time points; the amount of miR156 in hda6-7 hda9-1 was not significantly different from hda9-1 (Fig. 1d). To determine if HDA6 and HDA9 affected the level of miR156 by controlling the transcription of MIR156A/MIR156C, we examined the
Fig. 1 HDA6 and HDA9 act in miR156-dependent and miR156-independent pathways to promote vegetative phase change in Arabidopsis. (a) Rosettes and successive leaves of Col, hda6-7, hda9-1, and hda6-7 hda9-1 grown under short-day (SD) conditions. Bar, 3 mm. (b) hda6-7 and hda9-1 delay the production of abaxial trichomes in SDs. (c) The leaf blade length : width ratio in leaf 1, leaf 3, leaf 5 and leaf 7 of Col and mutants. (d) RT-qPCR analysis of temporal variation of miR156 in the shoot apices of Col and mutants. Values are relative to Col at day 8 and represent the mean ± SE from three biological replicates. *, Significant difference between hda9-1 and Col, hda6-7 hda9-1 and hda6-7, P < 0.05, one-way analysis of variance (ANOVA). ns, no significant difference between hda6-7 and Col, P > 0.05, one-way ANOVA. (e) RT-qPCR analysis of pri-miR156a, pri-miR156c, SPL9 and SPL15 in Day 20 shoot apices of Col and mutants. Shared letters indicate not significantly different groups, different letters indicate significantly different groups, P < 0.05, one-way ANOVA. Comparison in (e) was performed within groups.
primary transcripts of these loci (pri-miR156a and pri-miR156c). Consistent with the results for miR156, pri-miR156a and pri-miR156c were significantly elevated in hda6-7 (Fig. 1e). Among the 10 miR156-targeted SPLs in Arabidopsis, SPL9 and SPL15 are important for vegetative phase change. These genes are repressed by miR156 through both transcript cleavage and translational repression (Xu et al., 2016a; He et al., 2018). SPL9 transcripts were significantly reduced in both hda6-7 and hda9-1, and were reduced more in hda6-7 hda9-1 than in the single mutants (Fig. 1e). However, transcripts of SPL15 were downregulated in hda9-1 but not in hda6-7, and were downregulated to similar levels in the hda9-1 single mutant and the hda6-7 hda9-1 double mutant (Fig. 1e). These results suggested that HDA6 and HAD9 both regulated vegetative phase change, but through different mechanisms. HDA9 repressed the transcription of MIR156A/MIR156C, while HDA6 directly or indirectly promoted the transcription of SPL9 independently of miR156, and may also regulate the downstream targets of SPL proteins.

HDA9 binds to MIR156A and MIR156C in a temporally regulated manner

To determine how HDA6 and HDA9 regulate vegetative phase change, we examined the expression patterns of the HDA6 and HDA9 transcripts, and whether these proteins were recruited to miR156 and SPL genes. The abundance of HDA6 and HDA9 transcripts was not significantly different in 1-wk-old to 3-wk-old Col shoot apices (Fig. S1a), indicating that the regulation of vegetative phase change by them is not due to the transcriptional regulation of HDA6 and HDA9. To investigate the chromatin-binding patterns of these proteins, genomic constructs of HDA6::HDA6 fused to HA (HDA6::HDA6-HA) and HDA9::HDA9-GFP were transformed into hda6-7 and hda9-1, respectively, and transgenic lines in which these mutations were complemented were selected for further analysis (Fig. S2a,c,e,g). We used ChIP followed by qPCR (ChIP-qPCR) to examine if HDA6 bound to SPL9, as suggested by our observation that transcripts of SPL9 are decreased in hda6-7. We observed no binding of HDA6 to the SPL9 promoter or the region between the TSS and the second exon in 1-wk-old, 2-wk-old and 3-wk-old shoot apices (Fig. S1b,c).

We next examined the binding of HDA9 to MIR156A/ MIR156C in 1-wk-old, 2-wk-old and 3-wk-old shoot apices. We found that HDA9-GFP bound to the promoter and transcribed regions of MIR156A/MIR156C (Fig. 2a–d) and was more strongly associated with the region after the TSS than the region before the TSS. This was consistent with the observation that H3K27ac was more abundant in the region after the TSS than in the promoter region of these genes (Xu et al., 2016a). The association of HDA9 with MIR156A/MIR156C increased from 1 to 2 wk after planting, and declined from 2 to 3 wk. This was correlated with the greater decrease in H3K27ac from 1 to 2 wk after planting than from 2 to 3 wk (Fig. 2b,d) (Xu et al., 2016a). Together, our results indicated that HDA6 regulates SPL9 indirectly, independently of miR156, whereas HDA9 directly regulates MIR156A/MIR156C.

HDA9 associates with PKL to repress the transcription of MIR156A/MIR156C

PKL has activities in reducing H3K27ac levels at MIR156A/ MIR156C chromatin, although PKL itself is a chromatin remodeler (Xu et al., 2016a). To determine if PKL reduced H3K27ac levels through interacting with histone deacetylases, we investigated genetic interactions between these factors by crossing pkl-10 to hda6-7 and hda9-1. In SDs, the first leaf with the abaxial trichome in Col was leaf 7.5, whereas it was leaves 10, 11.1 and leaf 12.2 in hda6-7, hda9-1 and pkl-10, respectively. In the pkl-10 hda6-7 and pkl-10 hda9-1 double mutants, the first leaf with...
abaxial trichomes was leaf 21.5 and leaf 16.2, respectively (Figs 3a, S3a), indicating that PKL interacted synergistically with HDA6 and additively with HDA9. We then examined the levels of miR156 in these single mutants and double mutants. RT-qPCR showed that in 8-d-old plants miR156 was elevated c.50% in the pkl-10 and hda9-1 single mutants and by c.100% in the pkl-10 hda9-1 double mutant (Fig. 3b) and was reduced in both the single and double mutants as plants aged (Fig. 3b). By contrast, the level of miR156 in the pkl-10 hda6-7 double mutant was similar to the level in pkl-10 (l Fig. S3b). This was consistent with our observation that hda6-7 did not affect the level of miR156 (Fig. 1d). RT-qPCR analysis of the pri-miR156a transcript showed that Col, pkl-10, hda9-1 and pkl-10 hda9-1 had similar amounts of this transcript at 8 d after planting, but that it subsequently decreased more slowly in hda9-1, pkl-10 than in Col (Figs 3c, S3c,e). pri-miR156c was more abundant in pkl-10,
hda9-1 and pkl-10 hda9-1 than in Col at day 8, then declined in pkl-10 hda9-1 more slowly than in Col, pkl-10, or hda9-1 (Figs. 3d, S3d,f). To determine if this difference in miR156 levels was functionally significant, we measured the abundance of the transcripts of two of its targets, SPL9 and SPL15. These transcripts were reduced to 40–50% of the wild-type level in hda9-1 and pkl-10, and to 20–30% of the wild-type level in pkl-10 hda9-1 (Fig. 3e). These results demonstrated that PKL and HDA9 contributed to the downregulation of pri-miR156a and pri-miR156c.

To determine if PKL decreased H3K27ac levels at MIR156A/MIR156C by physically interacting with histone deacetylases, we tested the association of PKL with HDA6 and HDA9 using a Co-IP assay. Constructs of PKL::PKL-GFP, HDA6::HDA6-HA, and HDA9::HDA9-HA were transformed into pkl-10, hda6-7 and hda9-1, respectively, and transgenic lines in which the corresponding mutations were complemented were selected for further analysis (Fig. S2). PKL::PKL-GFP pkl-10 was crossed to HDA6::HDA6-HA hda6-7 and HDA9::HDA9-HA hda9-1, and plants homozygous for both transgenes and mutations were identified in F2 families derived from these crosses. Cell lysates of these lines were immunoprecipitated using an anti-GFP antibody, and GFP-associated proteins were subsequently subjected to western blot analysis using an anti-GFP antibody and an anti-HA antibody. HA-conjugated HDA6 or HDA9 were detected in the PKL::PKL-GFP HDA6::HDA6-HA pkl-10 hda6-7 and PKL::PKL-GFP HDA9::HDA9-HA pkl-10 hda9-1 samples, but not in the HDA6::HDA6-HA hda6-7 or the HDA9::HDA9-HA hda9-1 samples (Fig. 3f). This result suggested that PKL physically associates with HDA6 and HDA9 in planta.

HDA9 and PKL decrease H3K27ac and increase H3K27me3 and H2Aub at MIR156A/MIR156C

To determine how the PKL–HDA9 complex is involved in the transcriptional regulation of MIR156A/MIR156C, we examined the level of H3K27ac, H3K27me3 and H2Aub at these genes in the shoot apices of 1-wk, 2-wk and 3-wk-old wild-type plants and pkl-10, hda9-1 and pkl-10 hda9-1 mutants. Consistent with previous results (Xu et al., 2016a), the level of H3K27ac was highest at 1 wk and declined as plants aged. The 1-wk-old hda9-1 had higher levels of H3K27ac than Col at MIR156_A6 and MIR156_A7, and the pkl-10 hda9-1 double mutant had the highest level of H3K27ac at MIR156_A6, MIR156_A7 and MIR156_A10 (Fig. 4a–c). H3K27ac levels were significantly increased at MIR156_C7 and MIR156_C12 in 1-wk-old pkl-10 and hda9-1 single mutants, and the pkl-10 hda9-1 double mutant had the highest levels of H3K27ac at MIR156_C7, MIR156_C8 and MIR156_C12 (Fig. 4d). This suggested that both PKL and HDA9 contribute to erasing H3K27 acetylation at MIR156A/MIR156C, and they had a bigger effect on MIR156C than MIR156A.

Next, we asked if the elevated level of H3K27ac at MIR156A/MIR156C in pkl-10 and hda9-1 was correlated with a decreased level of H3K27me3 at these genes. We found that the level of H3K27me3 at MIR156_A6 was lower in 2-wk-old and 3-wk-old pkl-10 mutants than in wild-type plants, but that there was no significant difference in H3K27me3 at MIR156_A7 or at MIR156_A10 (Fig. 4e). The levels of H3K27me3 at MIR156_C7, MIR156_C8, and MIR156_C12 were lower than in the wild-type in 2-wk-old and 3-wk-old pkl-10 plants (Fig. 4f). The pkl-10 hda9-1 double mutant had a lower level of H3K27me3 than pkl-10 at MIR156_C7, MIR156_C8 and MIR156_C12 (Fig. 4e,f). These results suggested that PKL and HDA9 functioned similarly in modulating H3K27ac, but had different functions in regulating H3K27me3 at MIR156A/MIR156C. HDA9 seems to have no role in regulating H3K27me3 at MIR156A, but acts with PKL to regulate H3K27me3 at MIR156C. This may explain why pkl-10 hda9-1 had a larger effect on the expression of MIR156C than MIR156A.

Chromatin of MIR156A/MIR156C was also marked by H2A ubiquitination (H2Aub), which is catalysed by the activities of PRC1 (Picó et al., 2015; Fouracre et al., 2021). To determine if PKL and HDA9 affected the activities of PRC1, we examined the levels of H2Aub in Col, pkl-10, hda9-1 and pkl-10 hda9-1 mutants. An analysis of H2Aub levels in wild-type plants showed that H2Aub decreased from 1 to 2 wk at MIR156A, but increased from 1 to 3 wk at MIR156C (Fig. 4g,h). H2Aub levels were significantly lower in 2-wk-old pkl-10 or hda9-1 single mutants than in the wild-type at both MIR156A and MIR156C, and H2Aub levels were lowest in pkl-10 hda9-1 in 1-wk and 2-wk-old plants (Fig. 4g,h). These results indicated that PKL and HDA9 regulated H2Aub levels differently from the VAL1 transcription factor, which is uniformly present at MIR156A/MIR156C and regulated the overall amount of miR156 rather than the temporal decrease in miR156 during vegetative phase change (Fouracre et al., 2021). Our results suggested that PKL and HDA9 modulation of H2A ubiquitination via PRC1 may precede the binding of PRC2, or be independent of PRC2, because mutations in these genes reduced H2Aub levels in 1-wk-old plants, but did not affect the level of H3K27me3 at this stage (Fig. 4e–h).

To investigate if PKL and HDA9 affected the levels of H3K27ac, H3K27me3 and H2Aub globally, we examined the overall levels of these marks in wild-type, pkl-10, hda9-1 and pkl-10 hda9-1 in three biological replicates. Nuclear proteins were isolated from 2-wk-old plants and assayed on western blots using anti-H3K27ac, anti-H3K27me3, anti-H2Aub and anti-H3 antibodies. Consistent with other reports, H3K27ac was elevated significantly in hda9-1 and pkl-10 hda9-1 (Kim et al., 2016; Mayer et al., 2019; Zeng et al., 2020) but was not significantly affected by pkl-10 (Figs 4i, S3). The global levels of H3K27me3 were not significantly reduced in pkl-10 and hda9-1 (Figs 4i, S3). However, the global levels of H2Aub were significantly reduced in pkl-10, hda9-1 and pkl-10 hda9-1 (Figs 4i, S4). Together, our results indicated that the histone deacetylase HDA9 erases acetylation on H3K27 globally. The chromatin remodeler PKL modulates the level of H3K27ac at specific genes, rather than globally and both PKL and HDA9 play a role in modulating H2Aub levels across the genome.
Fig. 4 PKL and HDA9 act together to promote deacetylation and methylation of H3K27, and ubiquitination of H2A at MIR156A/MIR156C. (a, b) Genomic locations of the MIR156A (a) and MIR156C (b) sites analysed by chromatin immunoprecipitation-quantitative polymerase chain reaction (ChIP-qPCR). The miR156 hairpin is coloured light brown. (c, d), ChIP-qPCR analysis of H3K27ac levels in 1-wk-old, 2-wk-old and 3-wk-old shoot apices of Col, pkl-10, hda9-1 and pkl-10 hda9-1. (e, f), ChIP-qPCR analysis of H3K27me3 levels in the same tissue as in (c, d). (g, h), ChIP-qPCR analysis of H2Aub levels in the same tissue as in (c, d). Values (c–h) represent the mean ± SE from three biological replicates. a, significant difference between Col and the single mutants; b, significant differences between the single mutants and the double mutant. *P < 0.05, one tailed, paired t-test. (i) Western blot analysis of H3K27ac, H3K27me3 and H2Aub in 2-wk-old shoot apices of Col, pkl-10, hda9-1 and pkl-10 hda9-1. Folds compared with Col are normalised based on H3 levels. *, Significantly different from Col, P < 0.05, t-test. Values represent the mean ± SE from three biological replicates.
HDA9 acts to facilitate the binding of PRC2 at MIR156A/MIR156C

To investigate if PKL and HDA9 promoted the binding of PRC2 at MIR156A/MIR156C, we examined the binding of FIE to these genes in pkl-10 and hda9-1. FIE is a homologue of the Drosophila PRC2 component, Esc, and is encoded by a single gene in Arabidopsis (Deng et al., 2013). Previously, we found that FIE-HA (encoded by a FIE::FIE-HA transgene) was barely associated with MIR156A/MIR156C in 1-wk-old plants, but was present at these genes in 2-wk-old plants (Xu et al., 2016a). We therefore examined the level of FIE-HA in wild-type, pkl-10 and hda9-1 in 2-wk-old plants. ChIP-qPCR analysis showed that pkl-10 and hda9-1 significantly affected the binding of FIE-HA at MIR156_A6, but did not affect the binding of FIE-HA at the other MIR156A sites we examined (Fig. 5a,b). PKL and HDA9 promoted the efficient association of FIE-HA with MIR156C more broadly, including at MIR156_C5, which is located before the TSS, and at MIR156_C7, MIR156_C8, MIR156_C9, which are located after the TSS. These results are consistent with the RT-qPCR analysis that there was more upregulation of pri-miR156c than pri-miR156a in pkl-10 and hda9-1 mutants (Fig. 5c,d).

Discussion

PKL associates with histone deacetylases

The juvenile-to-adult vegetative phase transition is an important decision in a plant’s life cycle, as this developmental transition is associated with floral induction, immune response, herbivore resistance and grain development (Jiao et al., 2010; Mao et al., 2017; Wang et al., 2018; Hyun et al., 2019). miR156 is a master regulator for the juvenile phase and downregulation of miR156 promotes the juvenile-to-adult phase transition (Wu et al., 2009; Xu et al., 2016a). Understanding the mechanisms of how miR156 is transcriptionally downregulated and how this repression is maintained is important for understanding how vegetative phase change is promoted and maintained.

Our previous analysis showed that PKL modulates the MIR156A/MIR156C chromatin structure dynamically and PKL promotes the deacetylation and methylation of H3K27 at MIR156A/MIR156C during vegetative phase change (Xu et al., 2016a). Here, we show that PKL physically associates with two histone deacetylases, HDA6 and HDA9, to mediate the juvenile-to-adult transition in Arabidopsis. Our analysis of the H3K27ac levels at MIR156A/MIR156C showed that 1-wk-old hda9-1 had higher levels of H3K27ac than Col at both MIR156A and MIR156C, but that 1-wk-old pkl-10 had higher levels of H3K27ac than Col at MIR156C, not MIR156A, suggesting that PKL and HDA9 function overlappingly and distinctively. Nevertheless, the H3K27ac levels were highest in the pkl-10 hda9-1 double mutant at both MIR156A and MIR156C, suggesting that PKL function through HDA9 and other factors. PKL is a chromatin remodeler that modulates local chromatin structure to facilitate the recruitment of other factors (such as histone modifiers), and loss of PKL activity may result in the partial loss of the other factors’ activities. Our co-IP analysis showed that PKL associates with HDA6 and HDA9, and that it could associate with other histone deacetylases to regulate H3K27ac levels and vegetative phase change in Arabidopsis.

HDA9 physically associates with plant SANT domain protein PWR and WD-40 protein HOS15 (Chen et al., 2016; Kim et al., 2016; Mayer et al., 2019), and mutations in PWR and HOS15 resulted in delayed vegetative phase change (Cheng et al., 2021), suggesting the presence of a complex including PKL, HDA9, PWR and HOS15. A recent study indicated that cell division plays an important role in regulating the expression of MIR156C, and reported that HDA9 contributes to a cell division-dependent

Fig. 5 PICKLE and HDA9 facilitate the binding of FIE to MIR156A and MIR156C. (a, c) Genomic locations of the MIR156A (a) and MIR156C (c) sites analysed by ChIP-qPCR. The miR156 hairpin is coloured light brown. (b, d) Anti-HA ChIP analysis of FIE-HA binding to MIR156A (b) and MIR156C (d) in 2-wk-old Col, pkl-10 and hda9-1. Values represent the mean ± SE from three biological replicates. *, P < 0.05, one-way ANOVA.
The data that support the findings of this study are available in the supplementary material of this article.

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Author contributions

TH and MX planned and designed the research, performed experiments and analysed data. DM and VH performed genotyping experiments. MX wrote the manuscript. All authors read and approved the manuscript.

ORCID

Mingli Xu https://orcid.org/0000-0001-7997-573X

References

Aichinger E, Villar CB, Di Mambro R, Sabatini S, Köhler C. 2011. The CHD3 chromatin remodeler PICKLE and polycomb group proteins antagonistically regulate meristem activity in the Arabidopsis root. Plant Cell 23: 1047–1060.

Aichinger E, Villar CB, Farrona S, Reyes JC, Hennig L, Köhler C. 2009. CHD3 proteins and polycomb group proteins antagonistically determine cell identity in Arabidopsis. PLoS Genetics 5: e1000605.

Angel A, Song J, Dean C, Howard M. 2011. A polycomb-based switch underlying quantitative epigenetic memory. Nature 476: 105–108.
Leichty AR, Poethig RS. 2019. Development and evolution of age-dependent defenses in ant-accas. *Proceedings of the National Academy of Sciences*, USA 116: 15596–15601.

Li C, Chen C, Gao L, Yang S, Nguyen VI, Shi X, Siminovitch K, Kohalmi SE, Huang S, Wu K et al. 2015. The Arabidopsis SW2/SN2 chromatin remodeler BRAHMA regulates polycomb function during vegetative development and directly activates the flowering repressor gene SVP. *PLoS Genetics* 11: e1004944.

Li HC, Chuang K, Henderson JT, Rider SD Jr, Bai Y, Zhang H, Fountain M, Gerber J, Ogas J. 2005. PICKLE acts during germination to repress expression of embryonic traits. *The Plant Journal* 44: 1010–1022.

Mao YB, Liu YQ, Chen DY, Chen FY, Fang X, Hong GJ, Wang LJ, Wang JW, Chen XY. 2017. Jasmonate response delay and defense metabolite accumulation contributes to age-regulated dynamics of plant insect resistance. *Nature Communications* 8: 13925.

Mayer KS, Chen X, Sanders D, Chen J, Jiang J, Nguyen P, Salf M, Smith LM, Zhong X. 2019. HDA9-PWR-HOS15 Is a core histone deacetylase complex regulating transcription and development. *Plant Physiology* 180: 342–355.

Park HJ, Baek D, Cha YJ, Liao X, Kang SH, McClung CR, Lee SY, Yun DJ, Kim WW. 2019. HOS15 interacts with the histone deacetylate HDA9 and the evening complex to epigenetically regulate the floral activator GIGANTEA. *Plant Cell* 31: 37–51.

Pico S, Ortiz-Marchena MI, Merini W, Calonje M. 2015. Deciphering the role of POLYCOMB REPRESSIVE COMPLEX1 variants in regulating the acquisition of flowering competence in Arabidopsis. *Plant Physiology* 168: 1286–1297.

Poethig RS. 2003. Phase change and the regulation of developmental timing in plants. *Science* 301: 334–336.

Poethig RS. 2013. Vegetative phase change and shoot maturation in plants. *Current Topics in Developmental Biology* 105: 125–152.

Probst AV, Fagard M, Prous F, Mournain P, Boutet S, Earley K, Lawrence RJ, Pikaard CS, Murfett J, Furner I et al. 2004. Arabidopsis histone deacetylase HDA6 is required for maintenance of transcriptional gene silencing and determines nuclear organization of rDNA repeats. *Plant Cell* 16: 1021–1034.

Questa JL, Song J, Geraldo N, An H, Dean C. 2016. Arabidopsis transcriptional repressor VAIL1 triggers polycomb silencing at FLC during vernalization. *Science* 353: 485–488.

Tamada Y, Yun JJ, Woo SC, Amasino RM. 2009. *Arabidopsis thaliana* TRITHORAX-RELATED7 is required for methylation of lysine 4 of histone H3 and for transcriptional activation of FLOWERING LOCUS C. *Plant Cell* 21: 3257–3269.

Tanaka M, Kikuchi A, Kamada H. 2008. The Arabidopsis histone deacetylases HDA6 and HDA19 contribute to the repression of embryonic properties after germination. *Plant Physiology* 146: 149–161.

Telfer A, Bollman KM, Poethig RS. 1997. Phase change and the regulation of trichome distribution in Arabidopsis thaliana. *Development* 124: 645–654.

Tian R, Wang F, Zheng Q, Niaa V, Downie AB, Perry SE. 2020. Direct and indirect targets of the *arabidopsis* seed transcription factor ABC2111 ACID INSENSITIVE3. *The Plant Journal* 103: 1679–1694.

Turck F, Roudier F, Farrona S, Martin-Magnette ML, Guillaume E, Buisine N, Gagnon S, Martinssen RA, Coupland G, Colot V. 2007. *Arabidopsis TFL2/ LHP1* specifically associates with genes marked by trimethylation of histone H3 lysine 27. *PLoS Genetics* 3: e86.

Wang J, Zhou L, Shi H, Chen M, Yu H, Yi H, He M, Yin J, Zhu X, Li Y et al. 2018. A single transcription factor promotes both yield and immunity in rice. *Science* 361: 1026–1028.

Wang JW, Schwal B, Czech B, Mica E, Weigel D. 2008. Dual effects of miR156-targeted SPL genes and CYP78A5/KLUH on plastochron length and organ size in Arabidopsis thaliana. *Plant Cell* 20: 1231–1243.

Willmann MR, Poethig RS. 2007. Conservation and evolution of miRNA regulatory programs in plant development. *Current Opinion in Plant Biology* 10: 503–511.

Wood CC, Robertson M, Tanner G, Peacock WJ, Dennis ES, Hellwell CA. 2006. The *Arabidopsis thaliana* vernalization response requires a polycomb-like
protein complex that also includes VERNALIZATION INSENSITIVE 3. Proceedings of the National Academy of Sciences, USA 103: 14631–14636.

van der Woude LG, Perrella G, Snoek BL, van Hoogdalem M, Novák O, van Verk MC, van Kooten HN, Zorn LE, Tonckens R, Dongus JA et al. 2019. HISTONE DEACETYLASE 9 stimulates auxin-dependent thermomorphogenesis in Arabidopsis thaliana by mediating H2A.Z depletion. Proceedings of the National Academy of Sciences, USA 116: 25343–25354.

Wu B, Zhang M, Su S, Liu H, Gan J, Ma J. 2018. Structural insight into the role of VAL1 B3 domain for targeting to FLC locus in Arabidopsis thaliana. Biochemical and Biophysical Research Communications 501: 415–422.

Wu G, Park MY, Conway SR, Wang JW, Weigel D, Poethig RS. 2009. HISTONE DEACETYLASE 9 stimulates auxin-dependent histone H2A.Z trimethylation in Arabidopsis thaliana. Proceedings of the National Academy of Sciences, USA 106: 13820–13825.

Xu M, Hu T, Smith MR, Poethig RS. 2016a. Epigenetic regulation of vegetative phase change in Arabidopsis. Plant Cell 28: 28−41.

Xu M, Hu T, Zhao J, Park MY, Earley KW, Wu G, Yang L, Poethig RS. 2016b. Developmental functions of miR156-regulated SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes in Arabidopsis thaliana. PLoS Genetics 12: e1006263.

Xu M, Hu T, Poethig RS. 2021. Low light intensity delays vegetative phase change. Plant Physiology 187: 1177−1188.

Xu M, Leichty AR, Hu T, Poethig RS. 2018. H2A.Z promotes the transcription of MIR156A and MIR156C in Arabidopsis by facilitating the deposition of H3K4me3. Development 145: dev152868.

Xu Y, Guo C, Zhou B, Li C, Wang H, Zheng B, Ding H, Zhu Z, Peragine A, Cui Y et al. 2016. Regulation of vegetative phase change by SWI2/SNF2 chromatin remodeling ATPase BRAHMA. Plant Physiology 172: 2416–2428.

Yang C, Bratzel F, Hohmann N, Koch M, Turch F, Calonje M. 2013. VAL- and AtBMI1-mediated H2Aub initiate the switch from embryonic to postgerminative growth in Arabidopsis. Current Biology 23: 1324−1329.

Yang L, Chen X, Wang Z, Sun Q, Hong A, Zhang A, Zhong X, Hua J. 2020. HOS15 and HDA9 negatively regulate immunity through histone deacetylation of intracellular immune receptor NLR genes in Arabidopsis. New Phytologist 226: 507−522.

Yang L, Xu M, Koo Y, He J, Poethig RS. 2013. Sugar promotes vegetative phase change in Arabidopsis thaliana by repressing the expression of MIR156A and MIR156C. eLife 2: e00260.

Yu S, Cao L, Zhou CM, Zhang TQ, Lian H, Sun Y, Wu J, Huang J, Wang G, Wang JW. 2013. Sugar is an endogenous cue for juvenile-to-adult phase transition in plants. eLife 2: e00269.

van Zanten M, Zoll C, Wang Z, Philipp C, Carles A, Li Y, Kornet NG, Liu Y, Soppe WJ. 2014. HISTONE DEACETYLASE 9 represses seedling traits in Arabidopsis thaliana dry seeds. The Plant Journal 80: 475–488.

Zeng X, Gao Z, Jiang C, Yang Y, Liu R, He Y. 2020. HISTONE DEACETYLASE 9 functions with polycomb silencing to repress FLOWERING LOCUS C expression. Plant Physiology 182: 555−565.

Zhang H, Bishop B, Ringenberg W, Muir WM, Ogas J. 2012. The CHD3 remodeler PICKLE associates with genes enriched for trimethylation of histone H3 lysine 27. Plant Physiology 159: 418−432.

Zhang H, Rider SD, Henderson JT, Fountain M, Chuang K, Kandachar V, Simons A, Edenberg HJ, Romero-Severson J, Muir WM et al. 2008. The CHD3 remodeler PICKLE promotes trimethylation of histone H3 lysine 27. Journal of Biological Chemistry 283: 22637−22648.

Zhang X, Clarens O, Cokus S, Bernatavichute YV, Pellegrini M, Goodrich J, Jacobsen SE. 2007a. Whole-genome analysis of histone H3 lysine 27 trimethylation in Arabidopsis. PLoS Biology 5: e129.

Zhang X, Germann S, Blus BJ, Khorasanizadeh S, Gaudin V, Jacobsen SE. 2007b. The Arabidopsis LHP1 protein colocalizes with histone H3 Lys27 trimethylation. Nature Structural & Molecular Biology 14: 869−871.

Zhou Y, Romero-Campero FJ, Gómez-Zambrano A, Turch F, Calonje M. 2017. H2A monoubiquitination in Arabidopsis thaliana is generally independent of LHP1 and PRC2 activity. Genome Biology 18: 69.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 HDA6 does not bind to SPL9 directly.

Fig. S2 Complementation assay of transgenic plants.

Fig. S3 Genetic interaction between PICKLE (PKL) and HDA6, and PKL and HDA9.

Fig. S4 Western blot analysis of H3K27ac, H3K27me3 and H2Aub in 2-wk-old shoot apices of Col, hda9-1, pkl-10 and pkl-10 hda9-1 in two biological replicates.

Table S1 Primers used in this study.

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