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RESEARCH ARTICLES

Arabidopsis Relatives of the Human Lysine-Specific Demethylase1 Repress the Expression of *FWA* and *FLOWERING LOCUS C* and Thus Promote the Floral Transition [W]

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The timing of the developmental transition to flowering is critical to reproductive success in plants. Here, we show that *Arabidopsis thaliana* homologs of human Lysine-Specific Demethylase1 (LSD1; a histone H3-Lys 4 demethylase) reduce the levels of histone H3-Lys 4 methylation in chromatin of the floral repressor *FLOWERING LOCUS C* (*FLC*) and the sporophytically silenced floral repressor *FWA*. Two of the homologs, *LSD1-LIKE1* (*LDL1*) and *LSD1-LIKE2* (*LDL2*), act in partial redundancy with *FLOWERING LOCUS D* (*FLD*; an additional homolog of *LSD1*) to repress *FLC* expression. However, *LDL1* and *LDL2* appear to act independently of *FLD* in the silencing of *FWA*, indicating that there is target gene specialization within this histone demethylase family. Loss of function of *LDL1* and *LDL2* affects DNA methylation on *FWA*, whereas *FLC* repression does not appear to involve DNA methylation; thus, members of the LDL family can participate in a range of silencing mechanisms.

INTRODUCTION

The developmental transition from a vegetative to a reproductive phase (i.e., flowering) is perhaps the most critical event in the plant life cycle. In *Arabidopsis thaliana*, several pathways form a regulatory network that integrates the endogenous development state of the plant and environmental cues (e.g., daylength and temperature) to control the timing of the initiation of flowering (Mouradov et al., 2002; Putterill et al., 2004; Balasubramanian et al., 2006).

A key component in this regulatory network in *Arabidopsis* is FLOWERING LOCUS C (FLC), a MADS box transcription factor that blocks the floral transition (Michaels and Amasino, 1999; Sheldon et al., 1999). Therefore, repression of *FLC* expression results in the acceleration of flowering. The vernalization pathway represses *FLC* in response to a prolonged cold exposure, whereas the autonomous pathway, which includes *FLOWERING LOCUS D* (*FLD*) and *FVE*, constitutively represses *FLC*. *FRIGIDA* (*FRI*) activates *FLC* expression such that in the absence of vernalization, flowering is delayed (i.e., *FRI* establishes a vernalization requirement) (reviewed in Boss et al., 2004; Sung and Amasino, 2005).

Recent studies have revealed that chromatin modification plays an important role in the regulation of *FLC* expression. Histone H3 trimethylation at Lys-4 (H3K4me3) and histone acetylation are associated with active *FLC* transcription, whereas histone deacetylation and histone H3 methylation at Lys-9 (H3K9) and Lys-27 (H3K27) are associated with *FLC* repression (reviewed in He and Amasino, 2005). The autonomous-pathway repressors *FLD* and *FVE* are required for the deacetylation of *FLC* chromatin (He et al., 2003; Ausin et al., 2004; Kim et al., 2004). Vernalization leads to repressive histone modifications of *FLC* chromatin, including deacetylation, and increased methylation of H3K9 and H3K27 (Bastow et al., 2004; Finnegan et al., 2005; Sung et al., 2006). Activation of *FLC* expression and the associated increase in H3K4 trimethylation require the PAF1 (for RNA Polymerase II–Associated Factor1)–like complex (He et al., 2004).

FWA, a homeodomain-containing transcription factor first identified based on its ability to delay flowering, is also under epigenetic control. In wild-type *Arabidopsis*, *FWA* is silenced in the sporophyte; it is only expressed in female gamete and extraembryonic endosperm tissue in an imprinted (maternal origin–specific) manner (Soppe et al., 2000; Kinoshita et al., 2004). *fwa* epi-alleles (which do not have a change in the nucleotide sequence of *FWA*) cause a late-flowering phenotype due to ectopic *FWA* expression in sporophytic tissues (Soppe et al., 2000); ectopically expressed FWA interacts with FLOWERING LOCUS T and interferes with its function, delaying the floral transition (Ikeda et al., 2007). In the wild-type sporophyte, silent *FWA* chromatin is marked by repressive histone modifications and cytosine methylation in its 5′ region (Soppe et al., 2000; Lippman et al., 2004; Kinoshita et al., 2007). *Epi* alleles of *fwa* frequently arise in mutants defective in DNA methylation, such as *met1* (for *methyltransferase1*) (Saze et al.,

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2003), and in mutants defective in chromatin remodeling, such as *ddm1* (for *decreased DNA methylation1*) (Soppe et al., 2000).

Histone H3K4 methylation, which is associated with actively transcribed genes, plays an important role in regulating transcription (Martin and Zhang, 2005). The ε amino group of H3K4 residues can be monomethylated, dimethylated, or trimethylated. In budding yeast (*Saccharomyces cerevisiae*), trimethylated H3K4 is associated exclusively with active euchromatic genes (Santos-Rosa et al., 2002), whereas H3K4 dimethylation (H3K4me2) occurs in both inactive and active euchromatic genes (Ng et al., 2003). H3K4me2 is most prevalent in coding regions and the 3′ end of genes, and this pattern is thought to play a role in determining a transcriptionally permissive chromatin environment (Santos-Rosa et al., 2002; Ng et al., 2003). Similar to its association in *S. cerevisiae*, H3K4 trimethylation is associated with active transcribed genes in multicellular eukaryotes; however, in contrast with yeast, H3K4 dimethylation is also associated with active genes in multicellular eukaryotes (Schneider et al., 2004). For instance, active genes in chicken are marked with elevated levels of H3K4me2 and H3K4me3 at promoters and 5′ transcribed regions (Schneider et al., 2004). It has also been shown that in *Arabidopsis*, elevated levels of H3K4me2 and H3K4me3 are associated with active genes and that these modifications occur in 5′ promoters and coding regions but are absent from nontranscribed intergenic regions (Alvarez-Venegas and Avramova, 2005).

Histone H3K4 methylation is dynamically regulated by histone methylases and demethylases (Martin and Zhang, 2005). A component of transcriptional corepressor complexes, Lysine-Specific Demethylase1, has been shown to demethylate H3K4 and repress target gene expression in mammalian cells (Shi et al., 2004). Human LSD1 specifically demethylates monomethyl and dimethyl H3K4 (Shi et al., 2004; Forneris et al., 2005; Lee et al., 2005) and, when complexed with an androgen receptor, also destabilizes dimethyl H3K9 (H3K9me2) (Metzger et al., 2005). LSD1 is an integral component of several mammalian histone deacetylase (HDAC) corepressor complexes (Humphrey et al., 2001; Hakimi et al., 2002) in which HDACs and LSD1 may cooperate to remove activating acetyl and methyl histone modifications (Shi et al., 2005; Lee et al., 2006). Consistent with this model, in one such complex (the BRAF-HDAC complex), the enzymatic activities of HDACs and LSD1 are closely linked, as HDAC inhibitors diminish histone demethylation activity and the abrogation of LSD1 activity decreases the deacetylation activity of this complex (Lee et al., 2006).

We previously identified and characterized a plant homolog of human LSD1, FLD, which promotes flowering in *Arabidopsis* by constitutively repressing *FLC* expression (He et al., 2003). In this report, we demonstrate that *FLD* homologs, which we refer to as *LSD1-LIKE1* and *LSD1-LIKE2* (*LDL1* and *LDL2*), also contribute to the repression of *FLC* and that *LDL1* and *LDL2*, but not *FLD*, contribute to the sporophytic silencing of *FWA*.

RESULTS

Arabidopsis Has Four Relatives of Human LSD1

LSD1 is evolutionarily conserved among multicellular eukaryotes (Shi et al., 2004). We previously reported that FLD is a plant homolog of a repressor complex component that was later designated LSD1 (He et al., 2003). In addition to FLD, *Arabidopsis* has three other homologs of LSD1: *LDL1* (At_*1g62830*), *LDL2* (At_*3g13682*), and *LDL3* (At_*4g16310*) (Figure 1). Among these LSD1 relatives, FLD, LDL1, and LDL2 show extensive similarity (the similarity between FLD and LDL1 is 74% over a 624–amino acid region, and the similarity between LDL1 and LDL2 is 69% over a 739–amino acid region), whereas LDL3 shows less similarity to the other proteins (Figure 1).

LSD1 is a single-copy gene in the human genome (Shi et al., 2004), and the *Drosophila LSD1* homolog *SU(VAR)3-3* is also a single-copy gene (Rudolph et al., 2007), whereas both *Arabidopsis* (a eudicot) and rice (*Oryza sativa*; a monocot) have four homologs of *LSD1* (Figure 2), indicating that these *LSD1*-like genes were likely to have been duplicated before the monocot–eudicot split. Phylogenetic analysis of LSD1 relatives from different organisms showed that LDL1, LDL2, and FLD form a separate cluster that is related to the cluster of LSD1 and SU(VAR)3-3 (Figure 2). *FLD* has been shown to repress *FLC* expression and thus to promote flowering (He et al., 2003); however, the roles of the *LDL*s are unknown.

LDL1 and *LDL2* Promote the Floral Transition

To elucidate the biological roles of these LSD1 relatives, we first identified two loss-of-function mutants of *LDL1* and one of *LDL2* (Figure 3A). In long days (LD; 16 h of light and 8 h of dark), *ldl1* mutants flowered later than the wild-type Columbia (Col), whereas no phenotype was observed in the *ldl2* single mutant (Figure 3C; flowering is measured by the developmental criterion of the number of leaves formed, prior to flowering, from the primary apical meristem). The *ldl1-2 ldl2* double mutant flowered later than the *ldl1* single mutant, but otherwise it appeared normal (Figures 3B and 3C). The differences in flowering behavior in LD between the wild type, the *ldl1* single mutant, and the *ldl1 ldl2* double mutant were moderate but statistically significant (Table 1). There was no alteration in the rate of leaf initiation in this double mutant relative to Col (data not shown).

The Late-Flowering Phenotype of *ldl1 ldl2* Is Partially Dependent on *FLC*

FLD, a homolog of *LDL1* and *LDL2*, constitutively represses *FLC* expression (He et al., 2003). To examine whether *LDL1* and *LDL2* also contribute to *FLC* repression, we examined whether the late-flowering phenotype of *ldl1 ldl2* is altered by the introduction of an *flc* null mutation. *flc ldl1 ldl2* triple mutants flowered earlier than *ldl1 ldl2* but still slightly later than the *flc* single mutant in LD (Figure 3D). Thus, the late-flowering phenotype of *ldl1 ldl2* is partly dependent on *FLC*, indicating that *LDL1* and *LDL2* may also repress the expression of another floral repressor(s). We also quantified *FLC* transcripts in the *ldl* single mutants and the *ldl1 ldl2* double mutant using real-time quantitative PCR. Consistent with the flowering phenotypes, *FLC* was upregulated in *ldl1* and *ldl1 ldl2* mutants and remained unchanged in *ldl2* mutants (Figure 4A).

LDL1, *LDL2*, and *FLD* Are Partially Redundant in Repressing *FLC* Expression

As noted above, a component of the delayed flowering resulting from the loss of *LDL1* and *LDL2* is due to *FLC*. *FLD* also represses

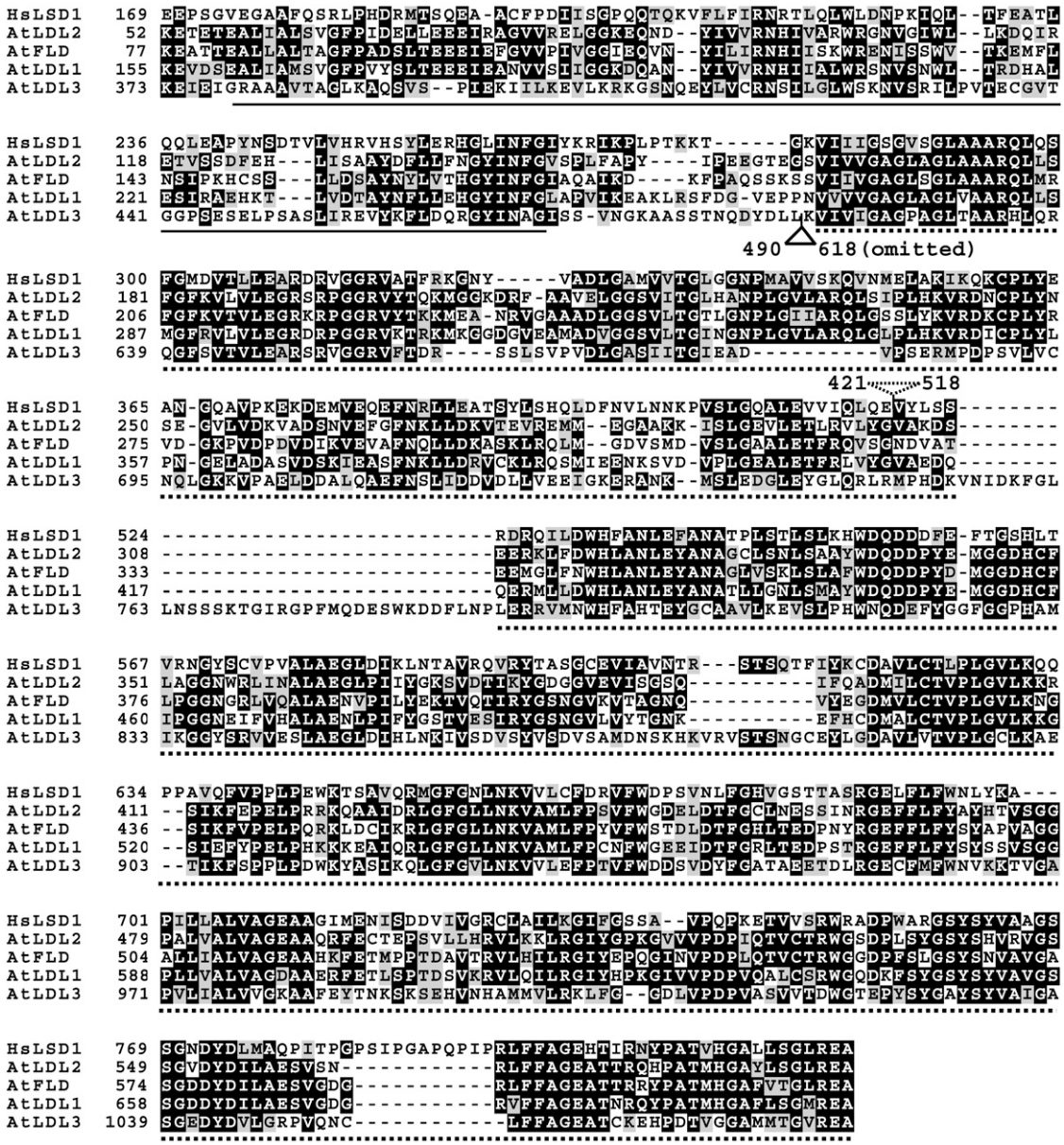

Figure 1. Amino Acid Sequence Alignment of *Arabidopsis thaliana* LDL1 (At LDL1), LDL2 (At LDL2), LDL3 (At LDL3), FLD (At FLD), and *Homo sapiens* LSD1 (Hs LSD1).

Numbers refer to amino acid residues; identical residues are shaded with black, and similar residues are shaded with gray. The SWIRM domain is indicated with a solid line; the conserved histone demethylation domain is indicated with a broken line. The broken triangle indicates the spacer region of Hs LSD1 (Shi et al., 2004) omitted from the alignment; the solid triangle indicates a 129–amino acid region of At LDL3 omitted from the alignment because it does not align with the rest of the proteins.

FLC expression; hence, it was of interest to examine the contributions of *FLD*, *LDL1*, and *LDL2* to the *FLC* expression level. As shown in Table 2, although the *fld* single mutant displays a strong late-flowering phenotype, the *ldl1 fld* double mutants flowered even later than *fld*, and this further delay in flowering was *FLC*-dependent (Figure 4B, Table 2). In addition, we quantified *FLC* transcript levels in seedlings of *ldl1-2*, *fld-3*, and *ldl1-2 fld-3* by real-time quantitative PCR. Consistent with the flowering phenotypes, *FLC* mRNA levels were higher in the *ldl1 fld* double mutant than in the *fld* single mutant (Figure 4B). Hence, *LDL1* and *FLD* are partially redundant in repressing *FLC* expression, with *FLD* playing a major role. *FLC* expression in the double mutants was still suppressed by vernalization (see Supplemental Figure 1 online), indicating that these genes are not part of the vernalization pathway in *Arabidopsis*.

The *ldl2* single mutation does not cause any flowering phenotype, and levels of *FLC* transcripts in *ldl2* remain the same as in Col (Figures 3C and 4A), but it is possible that *LDL2* contributes

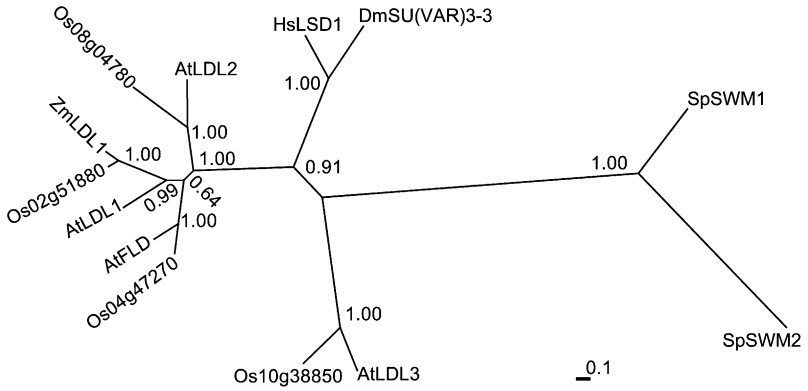

Figure 2. Phylogenetic Tree of LSD1 Relatives in Different Organisms.

The unrooted phylogram was generated using Mrbayes (version 3.1.2); amino acid sequences were aligned with ClustalW. At, *Arabidopsis thaliana*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Os, *Oryza sativa*; Sp, *Schizosaccharomyces pombe*; Zm, *Zea mays* (Zm LDL1, AZM4_71848; http://maize.tigr.org). Sp SWM1 (SPBC146.09c) and Sp SWM2 (SPAC23E2.02) are distant relatives of Hs LSD1. Clade credibility (posterior probability) values for each branch are shown.

redundantly with *LDL1* and *FLD* to *FLC* repression. Due to the chromosomal proximity of *LDL2* to *FLD*, it would be difficult to create the *fld ldl2* double mutant. Therefore, a double-stranded RNA interference approach using a 223-bp *LDL2*-specific fragment with no homology with *LDL1*, *LDL3*, or *FLD* was employed to knock down *LDL2* expression in *ldl1 fld* double mutants (in effect, mimicking a triple mutant). We quantified transcripts of *LDL2* and *FLC* in leaves of four independent T1 transgenic plants and found that in all transformants (which developed normally except for delayed flowering), *LDL2* expression was reduced and *FLC* expression was further elevated (Figure 4C). Thus, *LDL2* appears to act redundantly with *LDL1* and *FLD* to repress *FLC* expression.

FLC is preferentially expressed in shoot and root apical regions, which are enriched in dividing cells, and is also expressed in leaf vasculature (Bastow et al., 2004; Sung and Amasino, 2004). To examine whether *LDL1* and *LDL2* display a spatial expression pattern similar to that of *FLC*, we fused 5′ promoters and part of the coding regions of *LDL1* and *LDL2* with the reporter gene *GUS* (for β-*GLUCURONIDASE*). *LDL1* and *LDL2* were preferentially expressed in shoot and root apical regions of young seedlings (Figures 5A and 5B); in addition, *LDL1:GUS* was also expressed in vascular tissues of cotyledon leaves and was readily detectable in leaves of ~2-week-old plants (Figures 5A and 5C). Furthermore, we found that both *LDL1* and *LDL2* were expressed in inflorescences (Figures 5E and 5F).

LDL1 and *LDL2* Also Repress the Expression of *FWA*

As noted above, the delay in flowering of *ldl1 ldl2* double mutants appears to have an *FLC*-independent component. There is a close relative of *FLC* in *Arabidopsis*, *FLOWERING LOCUS M* (*FLM*), which is also a floral repressor (Scortecci et al., 2001). Because we and others previously found that both *FLC* and *FLM* are coordinately regulated by chromatin-modifying complexes such as the PAF1-like complex (He et al., 2004; Oh et al., 2004), it was of interest to determine whether *LDL1* and *LDL2* also repress *FLM* expression. We quantified *FLM* transcript levels in Col and *ldl1 ldl2* and found that *FLM* in *ldl1 ldl2* was expressed at a similar level to that in Col (see Supplemental Figure 2 online). Hence, unlike the PAF1-like complex, *LDL1* and *LDL2* repress the expression of *FLC* but not *FLM*.

We found, however, that expression of the homeodomain gene *FWA* was ectopically activated in rosette leaves of plants and seedlings of *ldl1* and *ldl2* single mutants and was even more elevated in the *ldl1 ldl2* double mutant (Figures 6A and 6B). *FWA* is normally silenced in the sporophyte but is expressed in extraembryonic tissue such as endosperm (Soppe et al., 2000; Kinoshita et al., 2004). Ectopic expression of *FWA* during sporophyte development (as occurs in *fwa* epi-alleles) leads to a delay in flowering (Soppe et al., 2000; Saze et al., 2003; Kinoshita et al., 2004). We examined *FWA* expression in seedlings of *ldl1-2 ldl2* and *met1-3* (a null allele in which *FWA* silencing is completely abrogated; Saze et al., 2003) and found that *FWA* transcript levels in *ldl1 ldl2* were lower than those in *met1* (Figure 6B), which is consistent with the weaker late-flowering phenotype of *flc ldl1 ldl2* relative to *met1*. We also examined the transcript levels of another heterochromatic locus, *Ta2* (a retrotransposon expressed at a low level during vegetative development; Mathieu et al., 2005), in *ldl1 ldl2* and found that, unlike the case for *FWA*, *Ta2* transcript levels in *ldl1 ldl2* were similar to those in Col (data not shown).

Because *FLD* is a homolog of *LDL1* and *LDL2*, we examined whether the loss of *FLD* also affected *FWA* expression. As in the wild type, *FWA* was not expressed in *fld* during vegetative development (Figure 6C); in addition, when an *fld* mutation was introduced into *ldl1-2* mutants, *FWA* expression was not enhanced (data not shown), which indicates that *FLD* is not involved in *FWA* repression. We also confirmed that the *FWA* activation in *ldl2* was indeed due to the *ldl2* lesion by transgenically rescuing the repression of *FWA* by introducing an *LDL2* construct (see Supplemental Figure 3 online). Thus, *LDL1* and *LDL2*, but not *FLD*, are required to silence *FWA* in the sporophyte.

We found no evidence for cross-regulation between *FLC* and *FWA*. In *fwa-1* epi-mutants (in which *FWA* is highly expressed), *FLC* mRNA levels were not altered relative to those in wild-type Landsberg *erecta* (data not shown). Also, as noted above, we did

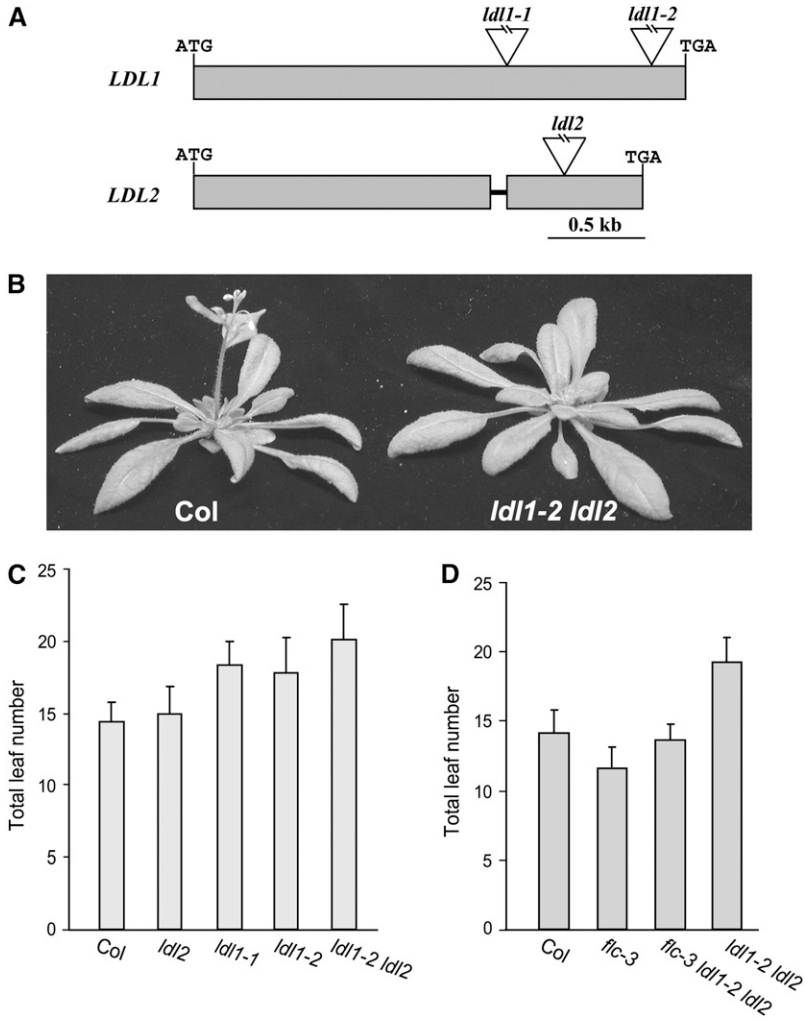

Figure 3. Phenotypes of *ldl1*, *ldl2*, and *ldl1 ldl2*.

(A) Gene structure of *LDL1* and *LDL2*. Exons are represented by closed boxes, and introns are represented by lines. Triangles indicate T-DNA insertions.

(B) Phenotypes of Col and the *ldl1 ldl2* mutant grown in LDs.

(C) Flowering times of *ldl1*, *ldl2*, and *ldl1 ldl2* mutants grown in LDs. The total number of primary rosette and cauline leaves at flowering was counted, and for each line at least 10 plants were scored. The values shown are means ± SD.

(D) Flowering times of *flc*, *ldl1 ldl2*, and *flc ldl1 ldl2* mutants grown in LDs. Total leaf number at flowering was scored. Fifteen plants were scored each for *flc*, *flc ldl1 ldl2*, and *ldl1 ldl2*; for Col, 10 plants were scored. The values shown are means ± SD.

not observe *FWA* activation in the *fld* mutant, in which *FLC* is highly expressed (Figure 6C).

LDL1 and *LDL2* Contribute to the de Novo Silencing of the *FWA* Transgene

When genomic clones of *FWA* are introduced into wild-type plants through *Agrobacterium tumefaciens*–mediated transformation, the *FWA* transgene is normally silenced and flowering in the transformed plants is not delayed (Chan et al., 2004, 2006a). To evaluate the role of *LDL*s in de novo silencing of the *FWA* transgene, we transformed *flc* and *flc ldl1 ldl2* mutants with the *FWA* transgene (Chan et al., 2004). As predicted from the results reported by Chan et al. (2004, 2006b), T1 transformants of *flc* mutants (predicted to behave like the wild type) flowered at a

Table 1. Student's *t* Test for the Flowering Times of *ldl1*, *ldl2*, and *ldl1 ldl2* Mutants in LDs

Value	Col versus *ldl1-1*	Col versus *ldl1-2*	Col versus *ldl2*	Col versus *ldl1-2 ldl2*	*ldl1-2* versus *ldl1-2 ldl2*	*ldl2* versus *ldl1-2 ldl2*
t value	6.42	4.75	0.75	8.38	3.03	7.43
P value	0.00	0.00	0.23	0.00	0.00	0.00

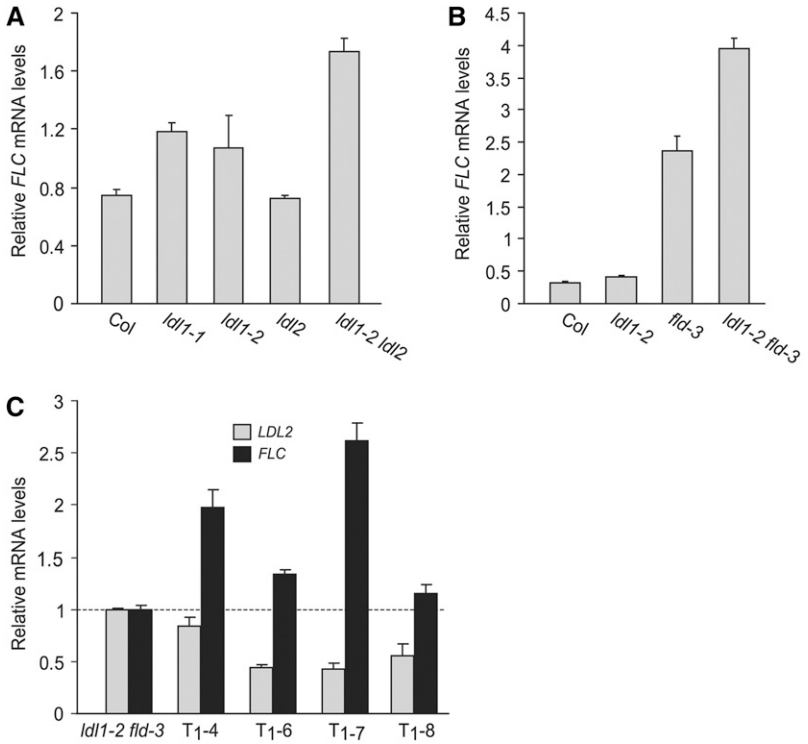

Figure 4. Repression of *FLC* by *LDL*s.

(A) Relative *FLC* mRNA levels in seedlings of *ldl1*, *ldl2*, and *ldl1 ldl2* quantified by real-time PCR.
(B) Relative *FLC* mRNA levels in seedlings of *ldl1*, *fld*, and *ldl1 fld* quantified by real-time PCR.
(C) Relative mRNA levels of *LDL2* and *FLC* in rosette leaves of *ldl1 fld* transformed with a double-stranded RNA interference construct targeting *LDL2*. Four independent T1 transgenic plants were examined; gray and black bars represent mRNA levels of *LDL2* and *FLC*, respectively.
The values shown are means ± SD.

similar time as *flc* (Figure 7). By contrast, T1 transformants of *flc ldl1 ldl2* mutants were late-flowering compared with nontransformed controls (Figure 7). Interestingly, the majority of T2 transgenic populations derived from self-pollination of the late-flowering T1 transformants flowered only slightly later than *flc ldl1 ldl2* (data not shown), which indicates that over a generation the *FWA* transgenes became partially silenced. Together, these results suggest that *LDL1* and *LDL2* play a role in the de novo silencing of *FWA*.

Around the start site of *FWA* transcription, there are two sets of tandem direct repeats derived from a retrotransposon that are part of a heterochromatin domain (Soppe et al., 2000; Lippman et al., 2004). These repeats produce short interfering RNAs (siRNAs) through the 24-nucleotide siRNA pathway, which work along with the chromatin-remodeling protein DRD1 (for DEFECTIVE IN RNA-DIRECTED DNA METHYLATION1) to de novo methylate *FWA* newly introduced into the *Arabidopsis* genome (Chan et al., 2004, 2006a). Therefore, we sought to examine whether the accumulation of *FWA* 24-nucleotide siRNAs was disrupted in *ldl1 ldl2* mutants. Previously, very low levels of *FWA* 24-nucleotide siRNAs were detected in wild-type plants (Lippman et al., 2004; Chan et al., 2006b; Kinoshita et al., 2007). Using radioactive probes derived from *FWA* tandem repeats, we detected low and similar levels of 24-nucleotide siRNAs in both Col and *ldl1 ldl2* seedlings (see Supplemental Figure 4 online), which indicates that *LDL1* and *LDL2* are not involved in the accumulation of *FWA* 24-nucleotide siRNAs.

Table 2. Total Leaf Number at Bolting for *ldl1*, *fld*, and *ldl1 fld* Mutants in LDs

Col	*ldl1-2*	*fld-3*	*ldl1-2 fld-3*	*flc-3*	*flc-3 ldl1-2 fld-3*
14.1 ± 1.7 (12)	16.1 ± 2.7 (15)	78.4 ± 10.8 (10)	>90.5 ± 3.6 (6)[a]	11.6 ± 1.6 (15)	14.9 ± 1.9 (15)

Values shown are means ± SD of total number of rosette and cauline leaves; numbers in parentheses indicate the number of plants scored.
[a] Two plants did not bolt in 3 months, and the mean number is the average of six plants scored.

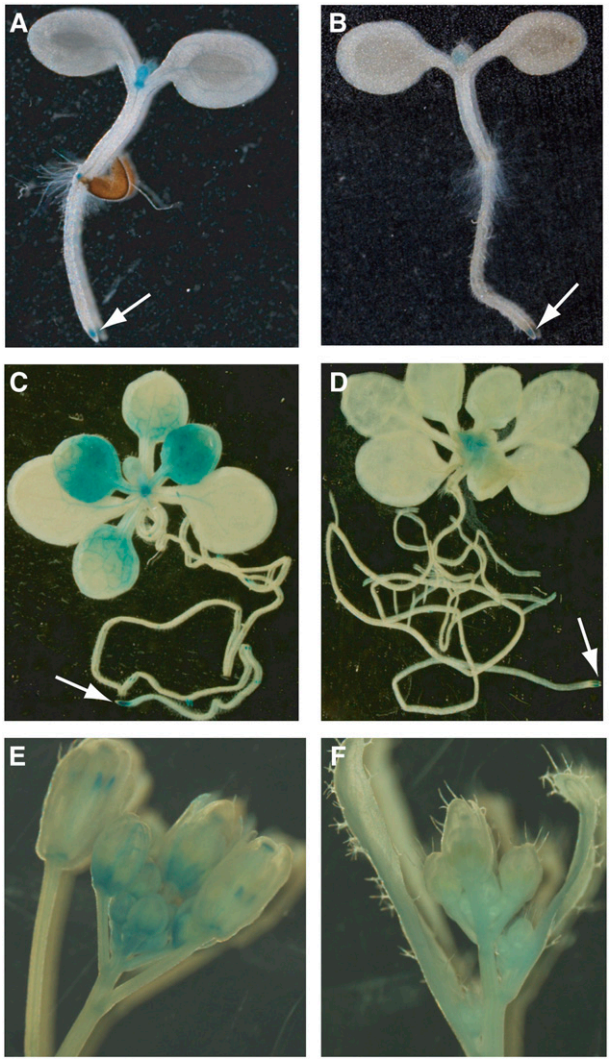

Figure 5. Histochemical Analysis of the Expression of *LDL1* and *LDL2*.

(A), **(C)**, and **(E)** Spatial expression patterns of *LDL1* in 4-d-old seedlings (T1), ~2-week-old plants (T2) grown on half-strength Murashige and Skoog medium, and inflorescence revealed by the *GUS* reporter gene driven by the *LDL1* promoter plus the 5′ part of *LDL1* CDS.
(B), **(D)**, and **(F)** Spatial expression patterns of *LDL2* in 4-d-old seedlings (T1), ~2-week-old plants (T2), and inflorescence revealed by *GUS* driven by the *LDL2* promoter plus the 5′ part of *LDL2* CDS.

LDL1, *LDL2*, and *FLD* Are Involved in H3K4 Methylation in Target Gene Chromatin

As noted above, LDL1, LDL2, and FLD are plant homologs of human LSD1 and *Drosophila* SU(VAR)3-3, which specifically demethylate monomethyl and dimethyl H3K4 (Shi et al., 2004; Forneris et al., 2005; Lee et al., 2005; Rudolph et al., 2007). H3K4me2 and H3K4me3 (presumably converted from H3K4me2) are often linked to gene transcription (Martin and Zhang, 2005). Hence, we evaluated the effect of the loss of LDL activities on the state of H3K4 methylation in the chromatin of *FWA* and *FLC* by chromatin immunoprecipitation (ChIP).

Previously, it was shown that the heterochromatin domain around the start site of *FWA* transcription, which is marked with repressive histone modifications and cytosine methylation, is involved directly in the silencing *FWA* in the sporophyte (Soppe et al., 2000; Lippman et al., 2004; Chan et al., 2006b). We first examined the chromatin state of the *FWA* heterochromatic domain in *ldl1 ldl2* using real-time quantitative PCR to quantify genomic fragments from seedlings after ChIP. Consistent with the activation of *FWA* in *ldl1 ldl2* seedlings (Figure 6B), the levels of H3K4me2 in *FWA* chromatin were increased and the levels of H3K4me3 were also moderately increased, whereas no obvious changes in the levels of H3K9me2 and H3K27me2 were observed in seedlings of *ldl1 ldl2* relative to Col (Figure 8B). We further assessed the H3K4me2 state of various *FWA* regions upstream and downstream of the heterochromatin domain. Immediately upstream, H3K4me2 was enriched in the 5′ promoter region in *ldl1 ldl2* relative to Col, whereas in regions 1.3 kb upstream and 1.7 kb downstream, H3K4me2 levels were similar in *ldl1 ldl2* compared with Col (Figure 8C). Hence, LDL1 and LDL2 regulate H3K4 methylation only around and within the heterochromatin domain in *FWA*. Therefore, LDL1 and LDL2 are involved in controlling the state of H3K4 methylation in *FWA* chromatin, consistent with their putative functions as H3K4 demethylases.

We also examined the state of H3K4 methylation in *FLC* chromatin in *ldl1 ldl2* mutants. The levels of H3K4me3 in both *FLC-P1* and *FLC-P2* were increased in seedlings of *ldl1 ldl2* relative to Col (Figure 8D), which is consistent with the moderate derepression of *FLC* in *ldl1 ldl2* (Figure 4A). We further examined the state of H3K4 methylation in *FLC* chromatin in *ldl1 fld* mutants and found that H3K4me3 was highly enriched in *FLC-P2* (around the transcription start site) and was also increased in *FLC-P1* in *ldl1 fld* relative to Col (Figure 8E). The relative levels of H3K4me3

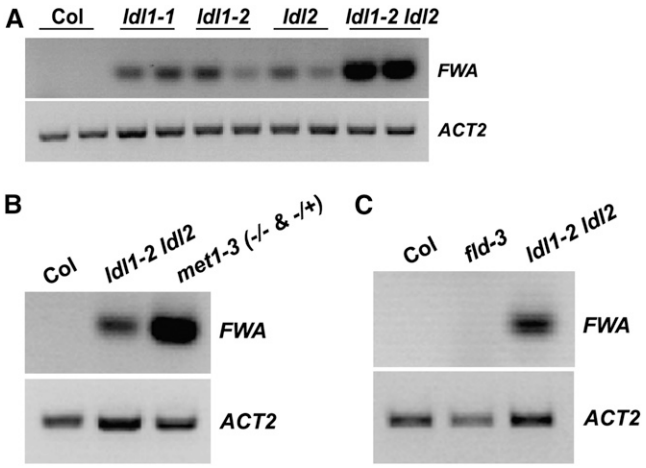

Figure 6. Derepression of *FWA* in *ldl1*, *ldl2*, and *ldl1 ldl2*.

(A) Ectopic activation of *FWA* in rosette leaves of *ldl1*, *ldl2*, and *ldl1 ldl2*. *FWA* transcripts were examined by RT-PCR, and duplicate lanes for each sample represent duplicate reactions. The constitutively expressed *ACTIN2* served as a control.
(B) Analysis of *FWA* expression in seedlings of *ldl1-2 ldl2* and *met1-3*.
(C) Analysis of *FWA* expression in rosette leaves of *fld*.

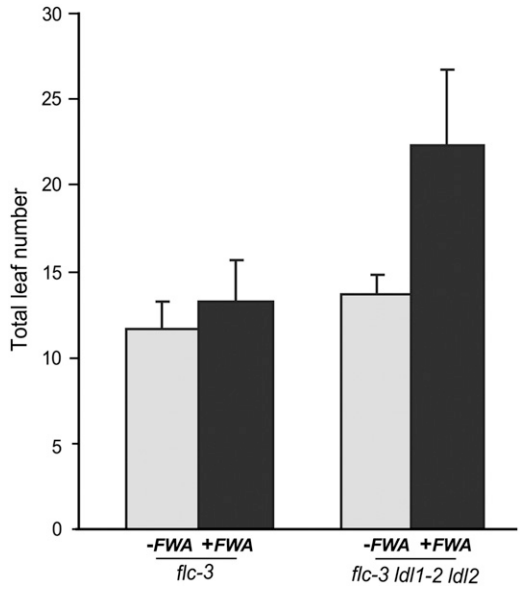

Figure 7. Flowering Times of *flc* and *flc ldl1 ldl2* Mutants Transformed with the *FWA* Transgene.

Plants were grown in LDs. Total leaf number at flowering was scored. Eighteen T1 transformants were scored for each transgenic population (*flc* and *flc ldl1 ldl2* as described in Figure 3D). The values shown are means ± SD.

in *FLC* in *ldl1 fld* compared with Col were higher than those in *ldl1 ldl2* compared with Col, indicating that *FLD* plays a more major role than *LDL1* and *LDL2* in regulating H3K4 methylation in *FLC* chromatin, consistent with the effects of lesions in these genes on flowering time and *FLC* expression. Furthermore, we found that the levels of H3K4me2 were also increased in a region in the 5′ promoter of *FLC* (*FLC-P1*), and surprisingly, we did not observe any change in the levels of H3K4me2 in *FLC-P2* in *ld11 fld* relative to Col (Figure 8E). It is possible that most of the H3K4me2 in *FLC-P2* may have been converted into H3K4me3, which is enriched in this region in *ldl1 fld*, as it was shown recently that a mammalian H3K4 methyltransferase specifically converts H3K4me2 into H3K4me3 in target gene chromatin (Hayashi et al., 2005). Together, these data indicate that *LDL1*, *LDL2*, and *FLD* are involved in controlling the H3K4 methylation levels of *FLC* chromatin.

LDL1 and *LDL2* Are Required for Full-Level DNA Methylation on *FWA*

DNA methylation on the heterochromatic region consisting of tandem direct repeats in the *FWA* locus is involved in its silencing (Soppe et al., 2000; Lippman et al., 2004; Chan et al., 2006b). Hence, it was of interest to examine the state of cytosine methylation of the endogenous *FWA* and the *FWA* transgenes introduced into *ldl1 ldl2* double mutants. Using a bisulfite PCR/restriction enzyme assay as described by Chan et al. (2006b), regions of the tandem repeats were amplified by PCR, followed by *Cla*I restriction digestion. CpG methylation protects *Cla*I restriction sites from bisulfite conversion, thus allowing digestion by *Cla*I after bisulfite treatment. In the control (*flc* mutant) background, endogenous *FWA* was methylated, whereas *FWA* had reduced methylation in *ldl1 ldl2 flc* mutants (Figure 9B). As predicted from the results reported by Chan et al. (2004, 2006b), cytosines in the context of CpG in *FWA* transgenes (in T1 transformants) were effectively de novo methylated in the *flc* background; by contrast, the majority of CpGs in *FWA* transgenes were not methylated in *ldl1 ldl2 flc* mutants (Figures 9B and 9C). Previously, it was shown that the establishment of CpG methylation on *FWA* transgenes is blocked in mutants in which DRM2 (for DOMAIN-REARRANGED METHYLASE2, a de novo DNA methyltransferase) is mutated (Cao and Jacobsen, 2002). It is noteworthy that in *ldl1 ldl2* double mutants, de novo CpG methylation is not affected as strongly as in *drm2*. Histone modifications often affect cytosine methylation at CpNG and at asymmetric sites (CpHH, where H is A, C, or T) (Aufsatz et al., 2002; Chan et al., 2005; Ebbs and Bender, 2006); therefore, we also determined the levels of methylated cytosines at these two sites in *FWA* transgenes using bisulfite genomic sequencing. Consistent with the previous findings of Chan et al. (2004, 2006b), ~40% of CpNG and 25% of CpHH in *FWA* transgenes were methylated in the control background, whereas both CpNG and CpHH methylation were nearly completely eliminated in *ldl1 ldl2 flc* (Figure 9C). Hence, *LDL1* and *LDL2* are essential for non-CpG methylation on *FWA* trangenes. Therefore, *LDL1* and *LDL2* not only are required for full-level DNA methylation on endogenous *FWA* but also are essential for de novo non-CpG methylation on *FWA* newly introduced into the *Arabidopsis* genome.

DISCUSSION

Our studies reveal that *Arabidopsis* relatives of the human histone demethylase *LSD1* (*LDL1*, *LDL2*, and *FLD*) reduce the levels of H3K4 methylation in *FWA* and *FLC* chromatin and act to repress the expression of these two genes. *FLD*, *LDL1*, and *LDL2* act in partial redundancy to repress *FLC* expression, whereas *LDL1* and *LDL2* act independently of *FLD* to repress *FWA*. That different members of this *Arabidopsis* histone demethylase family have different target preferences may represent specialization related to the different regulatory strategies governing *FLC* and *FWA* expression.

FLC is preferentially expressed in shoot and root apical regions throughout vegetative development, and the levels of *FLC* must be precisely controlled for specific flowering behaviors to be achieved. *FLC* is repressed by a pathway that monitors seasonal change (vernalization), and the vernalization-mediated repressed state of *FLC* is reset each generation (Boss et al., 2004; Sung and Amasino, 2005). Autonomous pathway regulators (e.g., *FLD* and *FVE*) constitutively repress, but do not silence, *FLC* expression (i.e., in the presence of these repressors, *FLC* is still expressed at a low level) (Michaels and Amasino, 2001). Furthermore, DNA methylation does not appear to play a direct role in *FLC* regulation (Finnegan et al., 2005). Our studies show that *FLC* is repressed by *FLD* and its close homologs *LDL1* and *LDL2*. These putative H3K4 demethylases may be part of a corepressor complex involved in *FLC* repression.

While this article was in preparation, Krichevsky et al. (2007) reported that *SWP1* (for SWIRM domain PAO protein1), the gene

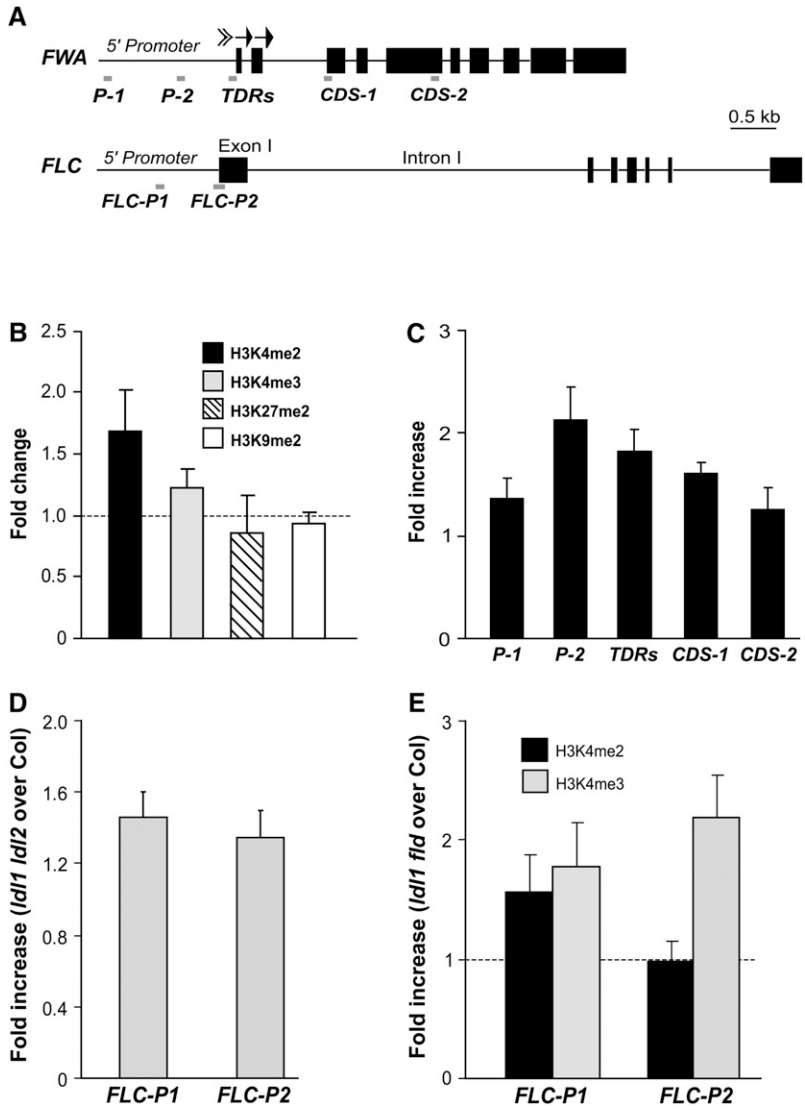

Figure 8. The Chromatin State of *FLC* and *FWA* in Col and *ldl* Mutants.

(A) Schematic structure of genomic *FWA* and *FLC* and the regions analyzed by real-time quantitative PCR after ChIP. The arrows represent two sets of tandem direct repeats in *FWA*.

(B) Histone methylation state of the heterochromatin domain in *FWA* chromatin in *ldl1 ldl2* and Col seedlings analyzed by ChIP. Each of the immunoprecipitations was performed at least three times. The immunoprecipitated DNA (corresponding to region *TDRs*) was quantified by real-time PCR and subsequently normalized to an internal control (*ACTIN2*). The fold changes of *ldl1 ldl2* over Col (i.e., the ratio of *ldl1 ldl2* to Col) are shown, and the values shown are means ± SD.

(C) H3K4me2 state of various regions in genomic *FWA* in *ldl1 ldl2* and Col. The fold enrichments of *ldl1 ldl2* over Col are shown, and the values shown are means ± SD.

(D) H3K4me3 state in *FLC* chromatin in *ldl1 ldl2* and Col seedlings. The fold enrichments of *FLC* in *ldl1 ldl2* over Col are shown, and the values shown are means ± SD.

(E) H3K4 methylation state in *FLC* chromatin in *ldl1 fld* and Col. Black and gray bars represent enrichments of H3K4me2 and H3K4me3, respectively. The fold enrichments of *ldl1 fld* over Col are shown, and the values shown are means ± SD.

referred to herein as *LDL1*, represses *FLC* expression. They reported that SWP1/LDL1 interacts with the putative H3K9 methyltransferase SUVR5/CZS [for Su(var)3-9–Related5/C2H2 zinc finger-SET domain HMT] and that lower levels of H3K9me2 around the transcription start site of *FLC* are detected in the wild-type Col but not in *swp1/ldl1* and *suvr5/czs* mutants. Other studies did not reproducibly detect H3K9me2 above the ChIP background levels in the 5′ region of *FLC* (upstream of the start codon) in *Arabidopsis* accessions such as Landsberg *erecta* and *FRI*-Col without vernalization treatment (Bastow et al., 2004; Liu

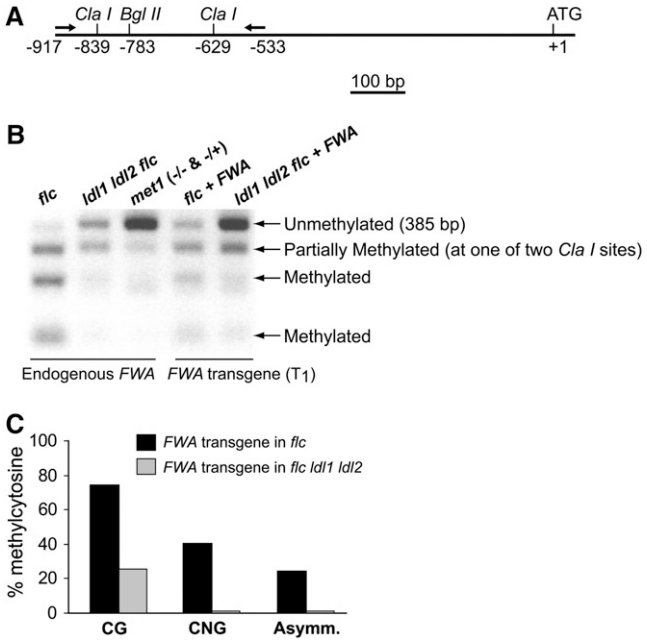

Figure 9. Methylation Patterns of Endogenous *FWA* and the *FWA* Transgene.

(A) Schematic drawing of 5′ *FWA*. Arrows depict the forward and reverse primers used to amplify the 5′ region of *FWA*.

(B) Methylation patterns of endogenous *FWA* and the *FWA* transgene. Genomic DNA was treated with bisulfite; subsequently, part of the tandem repeats in the 5′ region of *FWA* was amplified by PCR and followed by *Cla*I restriction digestion. CpG methylation prevents *Cla*I restriction sites from bisulfite conversion, thus allowing digestion by *Cla*I after bisulfite treatment. To analyze the *FWA* transgene, genomic DNA was first digested with *Bgl*II to destroy the endogenous *FWA*.

(C) *FWA* transgene (T1) cytosine methylation in CpG, CpNG, and asymmetric sites in *flc* and *flc ldl1 ldl2*.

et al., 2004; Sung and Amasino, 2004). We also examined the levels of H3K9me2 in the 5′ region of *FLC* in Col and *ldl1 ldl2* seedlings by ChIP-PCR and did not observe any difference in the levels of H3K9me2 between Col and *ldl1 ldl2* (see Supplemental Figure 5 online). The basis for these different results is not known, and it is intriguing that a SWP1/LDL-containing corepressor complex may work along with SUVR5 to generate a repressive chromatin environment at *FLC*.

As described above, *FLC* is repressed, but not silenced, in *Arabidopsis* lacking *FRI*. In contrast with *FLC*, *FWA* is effectively silenced in the sporophyte (as noted in Figure 6A, we could not detect *FWA* transcripts in sporophyte tissues after 40 cycles of PCR). Sporophytic silencing of *FWA* requires cytosine methylation (catalyzed by the MET1 methyltransferase at CpG sites) of the heterochromatin domain consisting of tandem direct repeats in its 5′ transcribed region (Saze et al., 2003). In addition, DDM1, a SWI2/SNF2-like chromatin-remodeling enzyme, is also required for full-level cytosine methylation on *FWA* (Jeddeloh et al., 1999; Soppe et al., 2000). *FWA* is ectopically activated in both *met1* and *ddm1* mutants because of the disruption of the maintenance of cytosine methylation and heterochromatin at the *FWA* locus (Soppe et al., 2000; Saze et al., 2003).

Our studies show that *LDL1* and *LDL2* are also required to maintain the silencing of endogenous *FWA*. Recently, it was shown that the *Drosophila* LSD1 homolog not only represses the expression of euchromatic genes but also functions in heterochromatin formation during embryonic development by blocking the expansion of H3K4 methylation from euchromatin into heterochromatin (Rudolph et al., 2007). *Drosophila* heterochromatin is defined by repressive histone modifications (Ebert et al., 2006), and CpG methylation is absent in *Drosophila* (only low amounts of CpT/A methylation have been detected in young embryos) (Lyko et al., 2000). *Arabidopsis* heterochromatic regions are often marked with cytosine methylation (mainly CpG methylation) in addition to repressive histone modifications (Chan et al., 2005). Although heterochromatic regions in these two organisms bear different features, our observations that H3K4me2 is enriched in and around the *FWA* heterochromatin domain in *ldl1 ldl2* relative to the wild type (Figure 8C) and that cytosine methylation in this domain is also partially lost in *ldl1 ldl2* (Figure 9B) lead us to speculate that LDL1 and LDL2 may also function in blocking the spread of H3K4 methylation from euchromatin to the heterochromatic region of endogenous *FWA* chromatin, and thus maintain this domain in a state of heterochromatin. It is intriguing that these putative histone demethylases may also be involved in maintaining DNA methylation on *FWA*. Recently, it was shown that the Polycomb group protein Enhancer of Zeste homolog2, a H3K27 methyltransferase, directly controls DNA methylation on target genes in mammalian cells (Vire et al., 2006). We speculate that removal of the activating histone methylations at H3K4 is perhaps required for DNA methyltransferases (e.g., MET1) to methylate cytosines in the heterochromatic *FWA* region.

De novo silencing of *FWA* newly introduced into the *Arabidopsis* genome through *Agrobacterium*-mediated transformation involves DNA methylation and heterochromatin formation directed by 24-nucleotide siRNAs and the chromatin-remodeling protein DRD1 (Chan et al., 2004, 2006a, 2006b). In this study, we have shown that *LDL1* and *LDL2* also play an important role in the de novo silencing of *FWA* newly introduced into *Arabidopsis*. Indeed, recent work in mammalian systems provides a framework for the role of LDLs in this process. DNMT3L (a mammalian DNA methyltransferase–like protein) binds specifically to histone H3 tails with unmethylated K4 and activates the DNMT3A DNA methyltransferase, resulting in de novo DNA methylation (Jia et al., 2007; Ooi et al., 2007). It is likely that the *FWA* transgene, newly introduced through *Agrobacterium*-mediated transformation, may acquire a chromatin state with active H3K4 methylation and that this H3K4 methylation must be removed for siRNA-directed de novo DNA methylation and silencing.

Human LSD1 is a component of several distinct corepressor complexes, such as the BRAF-HDAC complex (Hakimi et al., 2002) and the C-terminal binding protein complex (Shi et al., 2003). It is possible that different complexes with H3K4-demethylating activity are responsible for *FLC* and *FWA* repression and that different members of the plant H3K4 demethylase family are preferentially incorporated into different complexes.

That LDL1, LDL2, and FLD are close relatives of LSD1 (Figures 1 and 2) and that H3K4me2 is enriched in *FWA* chromatin in *ldl1*

ldl2 relative to the wild type indicate that these LDLs are likely to be H3K4 demethylases. Using recombinant FLD proteins purified from bacteria, we did not detect apparent demethylation activities of H3K4me2 and H3K9me2 with these proteins alone (data not shown). These in vitro enzymatic results are not surprising in light of the recent characterization of human LSD1 (Lee et al., 2005; Shi et al., 2005). Although recombinant LSD1 alone can demethylate H3K4 on free histones, the activities are much lower compared with those of LSD1-containing complexes (Lee et al., 2005); moreover, recombinant LSD1 is unable to demethylate H3K4 on nucleosomes, whereas the LSD1 complexes readily demethylate nucleosomes, indicating that cofactors associated with LSD1 stimulate its demethylation activities (Lee et al., 2005; Shi et al., 2005). In vivo LDL/FLD-containing complexes may possess the H3K4 demethylation activities; purifying these complexes from *Arabidopsis* will be essential to address the enzymatic characteristics of FLD/LDLs.

METHODS

Plant Materials

Arabidopsis thaliana ldl1-1, *ldl1-2*, and *ldl2* were isolated from the SALK collection (Alonso et al., 2003). *fld-3* (He et al., 2003), *flc-3* (Michaels and Amasino, 1999), *fwa-1* (Soppe et al., 2000), and *met1-3* (Saze et al., 2003) were described previously; pooled plants of *met1-3*/+ and *met1-3*/*met1-3* (selfed progeny of a *met1-3*/+ heterozygote) were used in this study, as *met1-3* homozygotes are genetically unstable.

RNA Isolation

Total RNAs from 10-d-old seedlings and expanded rosette leaves of adult plants were extracted with the RNeasy plant mini kit (Qiagen) or Tri reagent (Sigma-Aldrich) and were treated with RNase-free DNase according to the manufacturer's instructions (Qiagen).

Quantitative RT-PCR Assays

Real-time quantitative PCR was performed on an ABI Prism 7900HT sequence detection system using SYBR Green PCR master mix (Applied Biosystems). PCR proceeded as follows: 50°C (2 min), 95°C (10 min), and 40 cycles of 95°C (15 s) and 60°C (60 s); subsequently, a melting curve was generated to verify the specificity of the amplified fragment. Each sample was quantified at least in triplicate and normalized using *TUB2* (At_*5g62690*) as the control. Primers used are specified in Supplemental Table 1 online.

Analysis of *FWA* Transcripts in *ldl* by RT-PCR

Poly(A)$^+$ RNAs were purified from total RNAs extracted from rosette leaves of adult plants with the Oligotex kit according to the manufacturer's instructions (Qiagen). cDNAs were reverse-transcribed from poly(A)$^+$ RNAs with Moloney murine leukemia virus reverse transcriptase (Promega), and cDNAs of *FWA* were amplified in a 20-μL volume with 40 cycles of 94°C (30 s), 60°C (30 s), and 72°C (30 s). The primer pair used is specified in Supplemental Table 1 online.

siRNA Analysis by RNA Gel Blot

Using the Qiagen Midi kit, low-molecular-weight RNAs were isolated from total RNAs extracted with Tri reagent (Sigma-Aldrich). RNA gel blot analysis was performed as described previously (Kinoshita et al., 2007). Briefly, ~30 μg of RNAs was fractionated on a 17% polyacrylamide gel containing 7 M urea, transferred to a Hybond N$^+$ nylon membrane (Amersham) via electroblotting, and hybridized to ^{32}P-labeled probes covering the *FWA* repeat sequences (labeled via random priming) in PerfectHyb Plus buffer (Sigma-Aldrich). The 24-nucleotide RNA marker was described previously (Xie et al., 2004).

Plasmid Construction

To construct *LDL1-GUS*, a 1.3-kb *LDL1* genomic fragment including a 0.8-kb 5′ promoter plus a 0.5-kb coding region (CDS) was inserted into the pBGWFS7 vector (Karimi et al., 2005) via Gateway technology (Invitrogen); *LDL1* CDS was in-frame with the downstream *GUS* reporter gene, although they were not fused directly. To construct *LDL2-GUS*, a 3.5-kb *LDL2* genomic fragment including a 3.1-kb 5′ promoter plus a 0.4-kb CDS was inserted into the pBGWFS7 vector via Gateway technology; *LDL2* CDS was in-frame with the downstream *GUS* reporter gene.

Knockdown of *LDL2* via Double-Stranded RNA Interference

A 223-bp *LDL2*-specific fragment (from +1979 to +2201 of *LDL2* cDNA; the transcription start point was +1) was used to create a hairpin RNA by the AGRICOLA consortium (Hilson et al., 2004); the resulting binary plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 carrying *pMP90* and *pSOUP* helper plasmids through electroporation and subsequently was introduced into *ldl1-2 fld-3* mutants by the floral dip method (Clough and Bent, 1998).

ChIP and Real-Time Quantitative PCR Analysis

The ChIP experiments were performed as described previously (Johnson et al., 2002) using 10-d-old seedlings. Anti-dimethyl-histone H3 (Lys 4), anti-trimethyl-histone H3 (Lys 4), anti-dimethyl-histone H3 (Lys 27), and anti-dimethyl-histone H3 (Lys 9) were purchased from Upstate Biotechnology. The amounts of genomic DNA immunoprecipitated were determined by real-time quantitative PCR. Quantitative measurements of enrichments from *FWA* genomic regions and *ACTIN2* (At_*3g18780*) were performed on an ABI Prism 7900HT sequence detection system using TaqMan MGB probes (FAM dye–labeled) made by Applied Biosystems (the TaqMan gene expression assay identifier for *ACTIN2* was At02329915_s1) according to the manufacturer's instructions. Relative enrichments of various *FWA* regions in *ldl1 ldl2* over Col were calculated after normalization to *ACTIN2*; each of the immunoprecipitations was replicated at least three times (ChIP experiments with anti-dimethyl H3K4 were performed twice). Quantitative measurements of enrichments from regions of *FLC* and *TUB2* (At_*5g62690*) were performed using SYBR Green PCR master mix (Applied Biosystems); relative enrichments of *FLC* in *ldl1 fld* or *ldl1 ldl2* over Col were calculated after normalization to *TUB2*; each of the immunoprecipitations was repeated once, and each sample was quantified at least in triplicate.

Bisulfite PCR/Restriction Enzyme Assay and Bisulfite Genomic Sequencing

Approximately 2 μg of genomic DNA was treated with bisulfite as described previously (Soppe et al., 2000; Grunau et al., 2001); subsequently, part of the tandem repeats in the 5′ region of *FWA* (the bottom strand) was amplified by PCR, followed by *Cla*I restriction digestion as described by Chan et al. (2006b). Briefly, two nested pairs of primers (5′-CACCATTAATCCAAATACTATTTAATTATT-3′ and 5′-GGGATATTTATTGTAGAGTTAATATAATATTTTT-3′; 5′-CAAATACTATTTAATTATTTAAAATTACTTTTA-3′ and 5′-GGGAATTAAAATTATTTTTTAAATAAAATGTAAA-3′) were used to amplify *FWA*. PCR products were separated on an agarose gel, and the

fragments with the expected size were recovered from the gel and followed by *Cla*I digestion. To analyze the *FWA* transgene in T1 transformants of *flc* and *flc ldl1 ldl2* by *FWA*, genomic DNA was first digested with *Bgl*II to destroy the endogenous *FWA*; the *Bgl*II restriction site in the 5′ region of the *FWA* transgene was eliminated (Chan et al., 2006b). PCR fragments amplified from *FWA* transgenes (T1) were cloned into pGEM-T Easy vector (Promega) and sequenced further.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At*_1g62830* (*LDL1*), At*_3g13682* (*LDL2*), and At*_4g16310* (*LDL3*).

Supplemental Data

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

Supplemental Figure 1. Repression of *FLC* in *ldl1 fld* by Vernalization

Supplemental Figure 2. Relative *FLM* mRNA Levels in Col and *ldl1 ldl2* Seedlings Quantified by Real-Time PCR.

Supplemental Figure 3. Complementation of the *ldl2* Mutation.

Supplemental Figure 4. *FWA* siRNAs in Col and *ldl1 ldl2* Analyzed by RNA Gel Blotting.

Supplemental Figure 5. Levels of H3K9me2 in Col and *ldl1 ldl2* Seedlings Examined by ChIP-PCR.

Supplemental Table 1. Sequences of Primers Used in RT-PCR and ChIP-PCR Experiments.

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