The expression of long non-coding RNAs is associated with H3Ac and H3K4me2 changes regulated by the HDA6-LDL1/2 histone modification complex in *Arabidopsis*

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ABSTRACT

In recent years, eukaryotic long non-coding RNAs (lncRNAs) have been identified as important factors involved in a wide variety of biological processes, including histone modification, alternative splicing and transcription enhancement. The expression of lncRNAs is highly tissue-specific and is regulated by environmental stresses. Recently, a large number of plant lncRNAs have been identified, but very few of them have been studied in detail. Furthermore, the mechanism of lncRNA expression regulation remains largely unknown. *Arabidopsis* HISTONE DEACETYLASE 6 (HDA6) and LSD1-LIKE 1/2 (LDL1/2) can repress gene expression synergistically by regulating H3Ac/H3K4me. In this research, we performed RNA-seq and ChIP-seq analyses to further clarify the function of HDA6-LDL1/2. Our results indicated that the global expression of lncRNAs is increased in *hda6/ldl1/2* and that this increased lncRNA expression is particularly associated with H3Ac/H3K4me2 changes. In addition, we found that HDA6-LDL1/2 is important for repressing lncRNAs that are non-expressed or show low-expression, which may be strongly associated with plant development. GO-enrichment analysis also revealed that the neighboring genes of the lncRNAs that are upregulated in *hda6/ldl1/2* are associated with various developmental processes. Collectively, our results revealed that the expression of lncRNAs is associated with H3Ac/H3K4me2 changes regulated by the HDA6-LDL1/2 histone modification complex.

INTRODUCTION

High-throughput sequencing technologies have been widely used in the transcriptome analysis of eukaryotic systems. In previous studies, only ∼2% of eukaryotic genomes can be considered protein-coding regions. Many remaining transcripts with no protein-coding capacity are referred to as non-coding RNAs (ncRNAs) (1,2). Short-non-coding RNAs (<200 bp) comprise diverse groups, including transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), microRNA (miRNA) and small interfering RNA (siRNA). In recent years, long non-coding RNAs (lncRNAs, longer than 200 bp) have been identified as important regulators involved in diverse processes, including chromatin remodeling, histone modification, al-

ternative splicing and transcription activation (3–7). Generally, the expression of lncRNAs is lower than that of protein-coding genes and tends to be more tissue specific (8–12). In the human genome, only ∼11–29% of lncRNAs are ubiquitously expressed in all tissues, compared to 65% of protein-coding genes (1,13–14). Previous research has also revealed that the expression of plant lncRNAs is lower than that of animal lncRNAs (10). High diversity of lncRNA expression in different tissues and under different growth conditions has also been observed in plants (10,15).

LncRNAs have been identified as being associated with cancer and other diseases and are involved in various developmental processes in animals (4,8–9,11). In plants, it has been reported that lncRNAs are involved in root, flower and fruit development, light responses, vernalization, auxin transport and stress responses (15–29). For example, the *Arabidopsis* lncRNAs *cold-induced long antisense intragenic RNA* (*COOLAIR*) and *cold-assisted intronic noncoding RNA* (*COLDAIR*) are both transcribed from *flowering locus C* (*FLC*) and can be induced by cold treatment. Both *COOLAIR* and *COLDAIR* can repress the floral repressor *FLC* by interacting with Polycomb Repressive Complex 2 (PRC2) to increase the histone H3 lysine 27 trimethylation (H3K27me3) level of *FLC* (24–29). Another *Arabidopsis* lncRNA, *auxin-regulated promoter loop* (*APOLO*), can be activated by auxin and regulates the formation of a chromatin loop encompassing the promoter of the neighboring gene *Pinoid* (*PID*), which encodes an essential regulator of auxin polar transport (30). Although a large number of lncRNAs have been identified in *Arabidopsis,* very few of them have been studied in detail. It was found that decreased *FLC* expression is associated with an increased level of the repression marker H3K27me3 at the *FLC* locus during vernalization. In contrast, although H3K27me3 is increased near the chromatin locations of *COOLAIR* and *COLDAIR* during vernalization, their expression is not decreased but is increased (26–28), implying that H3K27me3 may function differently in *FLC* and *COOLAIR*/*COLDAIR*. In humans, it has been suggested that the general expression pattern of lncRNAs is not sensitive to changes in DNA methylation and H3K9me2 (31). These results suggest that the expression regulation of lncRNAs may be different from that of protein-coding genes. However, the mechanism of lncRNA expression regulation is rarely discussed and remains elusive in plants.

In yeast and animal systems, histone deacetylases (HDACs or HDAs) and lysine-specific demethylase 1 (LSD1) are the core components of several multiprotein HDAC complexes, such as Mi2/NuRD and CoREST. Class I HDACs and LSD1 act as the core factors in these complexes to repress gene expression by removing the methylation of histone H3 lysine 4 (H3K4me) and histone acetylation, both of which are active histone marks for transcription (32–36). *Arabidopsis* Histone Deacetylase 6 (HDA6) is an RPD3-like class I HDAC involved in various transcriptional regulation processes, including transposon repression and the regulation of ribosomal RNA (37–42). Furthermore, HDA6 is involved in flowering, leaf development, senescence, abiotic stress responses and circadian rhythms (43–47). There are four LSD1 homologs in *Arabidopsis*: LSD1-LIKE 1 (LDL1), LDL2, LDL3 and Flower-

ing Locus D (FLD) (48). LDL1 and LDL2 act redundantly to repress the expression of *FLC* by H3K4 demethylation. *ldl1/ldl2* double mutants also show reduced DNA methylation at the *FWA* locus, which represses the floral transition (48,49). Our recent studies have demonstrated that *Arabidopsis* HDA6 directly interacts with LDL1 and LDL2 in the same protein complex (46) and that they function synergistically to regulate histone H3 acetylation (H3Ac) and H3K4me in the core circadian clock genes (46,47).

In this research, we further analyzed the functions of HDA6 and LDL1 by characterizing the global H3Ac/H3K4me2 profiles and transcriptomes of wildtype (WT), *hda6*, *ldl1/ldl2* (*ldl1/2*) and *hda6/ldl1/ldl2* (*hda6/ldl1/2*). Furthermore, we compared the genomewide binding of HDA6 and LDL1 with these global H3Ac/H3K4me2 profiles and transcriptomes. Our results provide further details about the function of the *Arabidopsis* HDA6-LDL1/2 complex and reveal its involvement in the regulation of lncRNAs.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis (*Arabidopsis thaliana*) was grown in growth chambers under 12/12 h light/dark conditions at 22°C. In this study, the WT *Arabidopsis* Columbia (Col-0) ecotype was used. The mutants used in this research, including *ldl1/ldl2* (48), *hda6* (*axe1-5*) (45), *hda6/ldl1/2*, *LDL1pro::LDL1:GFP/ldl1* and *HDA6pro::HDA6:GFP/hda6* (46), were previously described.

RNA-seq analyses

For genome-wide expression analysis, RNA from 14-day-old seedlings of WT, *hda6*, *ldl1/2* and *hda6/ldl1/2* plants was isolated using the AllPure Plant RNA Purification Kit (Allbio, ABTGNA020-50) and treated with RNase-free DNase. RNA from at least three biological replicates was sequenced separately. Sequencing libraries were built using the Illumina TruSeq RNA library preparation protocol. The libraries were sequenced on the Illumina HiSeq 2500 platform using a paired-end scheme (2 × 150 bp) with TruSeq v3 chemistry. Reads were mapped to the TAIR10 *Arabidopsis* genome (50) using Qiagen CLC Genomics Workbench (QIAGEN, https://www.qiagenbioinformatics.com) with the default settings. The procedure of CLC Genomics Workbench data-processing was listed as following: Step 1, import fastaq.gz RAW data of the sequenced reads by *Standard Import* tool. Step 2, generate *metadata* to match samples and groups. Step 3, mapping reads to TAIR10 *Arabidopsis* genome (50) by *RNA-Seq Analysis* tool with default settings. Step 4, compare the reads with each gene between groups by *Differential Expression for RNA-Seq* tool with default settings. Step 5, export the computed reads and relative gene expression levels for further analysis. Genes exhibiting at least a 1.5-fold change in expression with a *P*-value < 0.05 were considered to be differentially expressed (Supplementary Table S1). The computed reads of all genes and the random selected coding gene lists were also listed in Supplementary Table S2 and 3.

To generate strand-specific bigWig files for visualization with the Integrated Genome Viewer (IGV) (51), raw sequencing reads were mapped to the *Arabidopsis* genome by usingTopHat2. The mapped reads were filtered with SAMtools (-F 0x4 -f 0x2), and only properly mapped read pairs were retained. To generate the strand-specific coverage files, the filtered reads were processed by bamCoverage with the following parameters: -bs 10, –normalizeUsing RPKM, reverse strand –samFlagInclude 64, –samFlagExclude 16; forward strand –samFlagInclude 80.

Quantitative reverse transcription PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, 15596026) according to the manufacturer's instructions. Two micrograms of DNase (Promega, RQ1 #M6101)-treated total RNA was used to synthesize cDNA (Promega, #1012891). RT-qPCR (real-time quantitative PCR) was performed using iQ SYBR Green Supermix solution (Bio-Rad, #170-8880). The CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) was used with the following cycling conditions: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, 60°C for 30 s and fluorescence detection. This was immediately followed by a melting curve (65–95°C in increments of 0.5°C for 5 s each with plate reading at each step). The melting curve analysis confirmed the absence of non-specific products. Each sample was quantified at least in triplicate and normalized by calculating the delta Cq (quantification cycle) value relative to the expression of the internal control *Ubiquitin10* (*UBQ10*). The Cq and relative expression values were calculated with Bio-Rad CFX Manager 3.1 based on the MIQE guidelines. The standard deviations were determined from at least three technical and two biological replicates. The variance of the average data is represented by the SEM (standard error of the mean). SDs (standard deviations), SEMs and *P*-values were calculated using Student's paired *t*-test. The gene-specific primers used for qRT-PCR are listed in Supplementary Table S4.

Chromatin immunoprecipitation assays

ChIP assays were performed as previously described (45). Whole 14-day -old seedlings grown under long-day conditions were collected and treated with 1% formaldehyde for chromatin-DNA extraction. The obtained chromatin DNA was sheared to a mean length of 500 bp by sonication, and proteins and DNA fragments were then immunoprecipitated using antibodies against acetylated histone H3K9K14 (Millipore, catalog no. 06-599), dimethylated histone H3K4 (Diagenode, catalog no. C15410035) or GFP (Abcam, catalog no. ab290). DNA crosslinking to the immunoprecipitated proteins was reversed, and the DNA was then analyzed by real-time PCR using specific primers (Supplementary Table S4). The percent input was calculated as follows: $2\wedge(Cq(IN)-Cq(IP))X100$, where Cq is the quantification cycle, as calculated by Bio-Rad CFX Manager 3.1 based on the MIQE guidelines. The SD were obtained from at least three technical and two biological replicates. The variance of the average data is represented by the SEM. SDs, SEMs and *P*-values were calculated using Student's paired *t*-test.

ChIP-seq and data analyses

ChIP-seq assays were performed based on previous research (46,52–55). The HDA6 ChIP-seq data (GSE132563) used in this study were proceed together with previously published LDL1 ChIP-seq data (GSE118025) (46). Samples of 5 ng of DNA from at least 5 ChIP replicates were pooled to ensure that there was enough starting DNA for library construction. Two biological replicates were prepared and sequenced for each ChIP-seq experiment. The ChIP DNA was first tested by qRT-PCR and then used to prepare ChIP-seq libraries. End repair, adaptor ligation and amplification were carried out using the Illumina Genomic DNA Sample Prep kit according to the manufacturer's protocol. An Illumina HiSeq 2500 instrument was used for the high-throughput sequencing of the ChIP-seq libraries. The raw sequence data were processed using the GAPipeline Illumina sequence data analysis pipeline. Bowtie2 was then employed to map the reads to the *Arabidopsis* genome (TAIR10) (50). Only perfectly and uniquely mapped reads were retained for further analysis. To determine the correlation between biological repeats, the Pearson correlation was computed using R statistical software according to the normalized signal intensity for ChIP binding peaks. The alignments were first converted to Wiggle (WIG) files using deepTools. The data were then imported into the Integrated Genome Viewer (IGV) (51) for visualization. The distribution of the ChIP binding peaks was analyzed with ChIPseeker (56), and a high-read random *Arabidopsis* genomic region subset (1 350 000 regions) was used to represent the ratio of the total *Arabidopsis* genomic regions. To identify DNA motifs enriched at LDL1-associated sites, 400-bp sequences encompassing each peak summit (200 bp upstream and 200 bp downstream) were extracted and searched for enriched DNA motifs using MEME-ChIP (57). Searches were performed using the default parameters. HDA6/LDL1-targeted lncRNAs were identified by comparing the HDA6/LDL1 binding sites with the lncRNA-associated genomic regions (15) (Supplementary Table S5). To compare the correlation between RNA expression and histone modifications, the ChIP-seq results as well as previously published *Arabidopsis* H3K4me3, H3K9me2, H3K27me3, H3K36me3, Pol II and Pol V ChIP-seq data (52,58–60) were analyzed by using deepTools to generate a correlation matrix among the different gene groups.

RESULTS

Genome-wide occupancy profiles of HDA6 and LDL1

To further investigate the function of the HDA6-LDL1/2 complex, we mapped the genome-wide occupancy of HDA6 by chromatin immunoprecipitation followed by sequencing (ChIP-seq). ChIP-seq analysis was carried out using transgenic plants expressing the HDA6:GFP fusion protein driven by the native *HDA6* promoter (*HDA6pro::HDA6:GFP*) in the *hda6* mutant background. The *HDA6:GFP* transgene can fully rescue the late-flowering phenotype of the *hda6* mutant, indicating that it is functional *in vivo* (Supplementary Figure S1). The ChIP-seq analyses identified 9,011 HDA6-targeted genomic regions (Figure 1A), including several regions located close to the
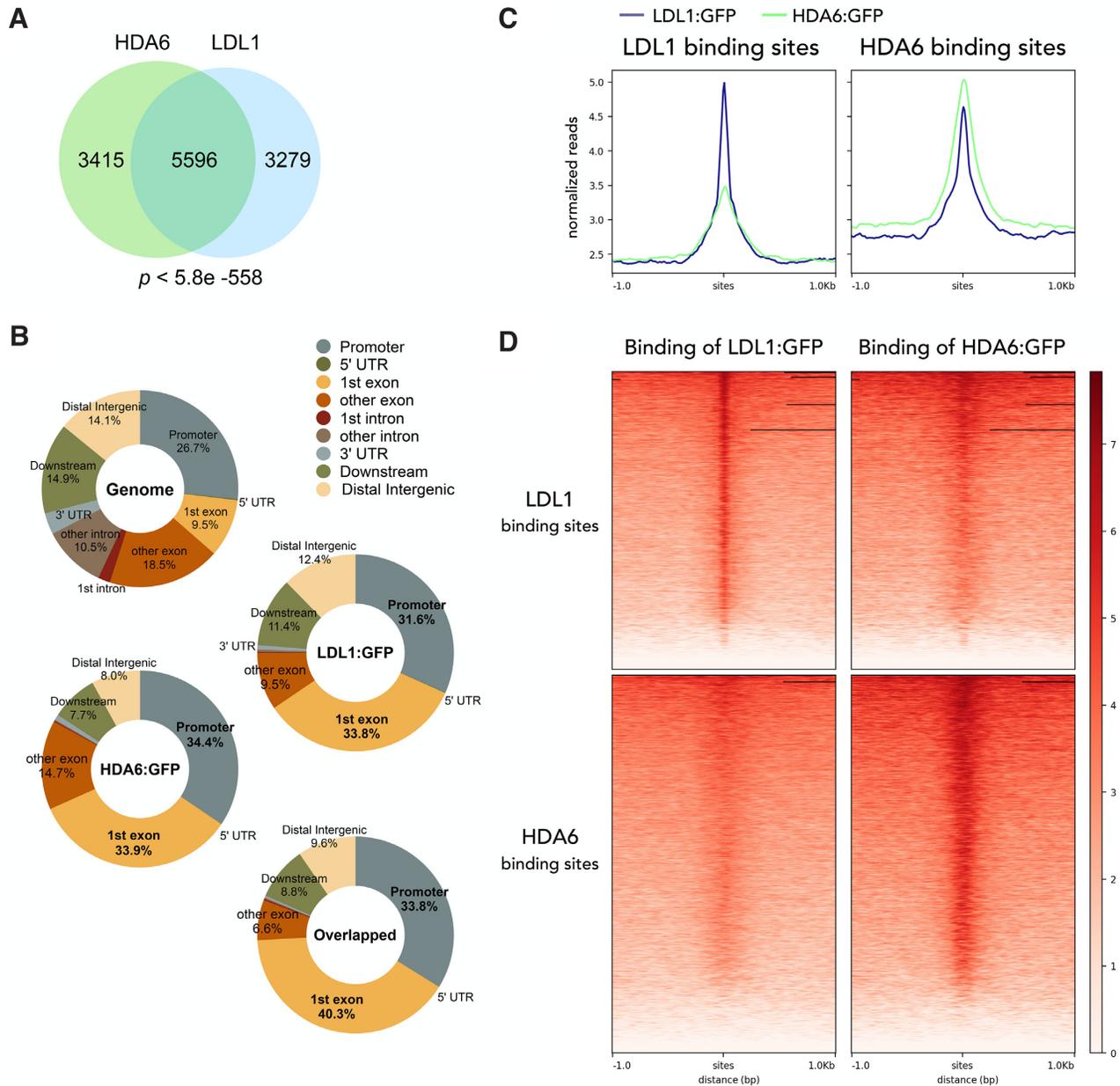
Figure 1. HDA6 and LDL1 show similar binding patterns in *Arabidopsis*. (**A**) A Venn diagram showing statistically significant overlap between HDA6 binding peaks and LDL1 binding peaks (5596 peaks; $P < 5.8e$ -558, hypergeometric test). (**B**) Pie charts showing the distribution of HDA6 and LDL1 in annotated genic and intergenic regions in the genome. (**C**) The mean density of HDA6 and LDL1 occupancy at all LDL1-associated sites or HDA6-associated sites. The average HDA6 and LDL1 binding signal within 1 kb genomic regions flanking the center of LDL1 or HDA6 peaks is shown. (**D**) Heat map representation of the cooccupancy of HDA6 and LDL1 in the genome. Each horizontal line represents an LDL1- or HDA6-bound region, and the signal intensity is shown for LDL1 binding and HDA6 binding (center). Columns show the genomic region surrounding each LDL1 or HDA6 peak. Signal intensity is indicated by the shade of red.

direct targets of HDA6 identified in previous studies (41–45). We previously reported the genome-wide occupancy profile of LDL1 (46). We further compared the genome-wide occupancy profiles of HDA6 and LDL1. We found that HDA6 and LDL1 cooccupy 5596 targeted genomic regions, indicating significant overlap between HDA6- and LDL1-occupied genomic regions (hypergeometric test, $P < 5.8e-558$) (Figure 1A). Compared to the *Arabidopsis* genomic region distribution, we found that the binding of

HDA6 and LDL1 was more enriched in the first exon and promoter regions but less enriched in UTR and intron regions (Figure 1B). Approximately 30–35% of HDA6- and LDL1-occupied sites are located in the promoters of protein-coding genes and 30–40% are located in the first exons of gene bodies (Figure 1B). Furthermore, the HDA6-enriched sites were located precisely at the LDL1-occupied sites, indicating that HDA6 and LDL1 show high similarity in their binding regions (Figure 1C and D). In addition,

many similar cis-elements were found to be enriched within the HDA6 and LDL1 binding sites, including $(GA/TC)_n$ repeats, G-boxes and TCP binding sites (TBSs) (Supplementary Figure S2). These results are consistent with the previous finding that HDACs and LSD1 function cooperatively in the same protein complex in *Arabidopsis* (33–36,46).

By comparing the genome-wide occupancy profiles of HDA6 and LDL1, we found that LDL1 is highly associated with HDA6-binding sites, but the binding of HDA6 at LDL1-binding sites is not as strong as that of LDL1 itself (Figure 1C and D). These results implied that although the function of LDL1 is highly correlated with that of HDA6, LDL1 may not always be associated with HDA6, suggesting that in addition to HDA6, other HDACs may also functionally associate with LDL1/2 in *Arabidopsis*.

The mechanism of expression regulation may be different between protein-coding genes and lncRNAs

We analyzed the distribution of the expression levels of lncRNAs and protein-coding genes in *Arabidopsis* and found that the expression of lncRNAs was lower than that of the protein-coding genes (Supplementary Figure S3A), which is consistent with previous studies in animals and plants (8–12). To eliminate analysis error, we created random expression-matched coding gene subsets for further analysis (Supplementary Figure S3A).

Histone modification marks are removable and may therefore provide a flexible strategy for gene repression or activation. H3Ac, H3K4me2/3 and H3K36me3 are active marks for transcription; in contrast, H3K9me2 and H3K27me3 are signals for transcriptional silencing (35). The data presented in recent studies have implied that the effect of H3K9me and H3K27me3 on lncRNAs may be different from that of protein-coding genes (26–28,31). However, the mechanism of lncRNA expression regulation is rarely discussed and remains elusive in plants. To further investigate the role of histone modifications in the regulation of lncRNA in *Arabidopsis*, we compared the H3Ac and H3K4me2 ChIP-seq results with previously published ChIP-seq results for H3K4me3, H3K9me2, H3K27me3 and H3K36me3 in WT *Arabidopsis* (52,58). We found that the expression of lncRNAs was positively correlated with H3Ac, H3K4me2/3 and H3K36me3 (Supplementary Figure S3B). However, the expression of lncRNAs was less correlated with H3K9me2 and H3K27me3 (Supplementary Figure S3B). Nevertheless, the expression of protein-coding genes still presented a negative correlation with H3K27me3 (Supplementary Figure S3B). These results indicated that the general expression of lncRNAs is less affected by H3K9me2 and H3K27me3 in the gene body of lncRNAs, implying that the effect of histone modifications may be different between protein-coding genes and lncRNAs.

RNA polymerase V (Pol V) is a plant-specific RNA polymerase that has an independent function from Pol II. Pol II is the most active RNA polymerase for protein-coding genes, small RNAs and rRNAs. In contrast, Pol V is highly associated with other non-coding RNA-mediated gene silencing processes, such as RNA-directed DNA methylation (RdDM) (61,62). A previous study suggested that the transcripts of specific lncRNAs are associated with Pol V (63). To further investigate the function of RNA polymerases in the regulation of lncRNA in *Arabidopsis,* we also compared the expression of lncRNAs with previously published ChIP-seq results for Pol II and Pol V in *Arabidopsis* (59,60). We found that the expression of lncRNAs was positively correlated with the enrichment of both Pol II and Pol V, in which the correlation between lncRNA expression and the enrichment of Pol V was relatively minor (Supplementary Figure S4). In contrast, the expression of protein-coding genes was positively correlated with the enrichment of Pol II but was not positively correlated with the enrichment of Pol V (Supplementary Figure S4). These results indicated that the regulation may be different between protein-coding genes and lncRNAs in *Arabidopsis*.

HDA6 and LDL1/2 are involved in the regulation of lncRNAs in *Arabidopsis*

To further investigate the regulatory targets of HDA6-LDL1/2, we performed RNA-seq analyses in WT, *hda6*, *ldl1/ldl2* (*ldl1/2*) and *hda6/ldl1/ldl2* (*hda6/ldl1/2*) mutant plants and identified differentially expressed genes (DEGs) from the TAIR10 *Arabidopsis* gene annotation. The upregulated and downregulated DEGs in *hda6*, *ldl1/2* and *hda6/ldl1/2* were analyzed among different gene types. Interestingly, we found that among all of the differentially expressed protein-coding genes identified in *hda6*, *ldl1/2* and *hda6/ldl1/2*, ~60% were upregulated and ~40% were downregulated. However, among all of the differentially expressed lncRNAs in *hda6* and *ldl1/2*, more than 74% were upregulated. Furthermore, more than 83% of the differentially expressed lncRNAs were upregulated in *hda6/ldl1/2* (Supplementary Figure S5), suggesting that HDA6-LDL1/2 may have greater effects on the expression of lncRNAs than that of protein-coding genes. The overall expression of all lncRNAs was higher in *hda6, ldl1/2* and *hda6/ldl1/2* than in WT (Figure 2A). In contrast, the expression patterns of the protein-coding genes were not substantially changed in *hda6*, *ldl1/2* and *hda6/ldl1/2* compared to WT (Figure 2A and Supplementary Figure S6A). We also compared the relative expression of protein-coding genes and lncRNAs. The fold change of lncRNAs was higher than that of protein-coding genes in *hda6* and *hda6/ldl1/2* compared to WT, and the largest increase in the expression of lncRNAs was observed in *hda6/ldl1/2* (Figure 2B). The general expression pattern showed no substantial difference between all coding genes and the expression-matched coding gene subsets (Supplementary Figure S6). However, the mean fold change observed for the total lncRNAs in *hda6/ldl1/2* was not greatly increased (Figure 2B), suggesting that HDA6-LDL1/2 may not regulate all of the lncRNAs, but only a specific fraction of them.

Interestingly, we also found that the levels of HDA6 and LDL1 binding within the promoters of lncRNAs were higher than within the protein-coding genes (Figure 2C). These results suggested that HDA6 and LDL1/2 may be highly associated with the regulation of lncRNAs. In addition, we performed H3Ac and H3K4me2 ChIP-seq analysis in WT, *hda6*, *ldl1/2* and *hda6/ldl1/2* to identify the effects of HDA6-LDL1/2 on histone modification and found
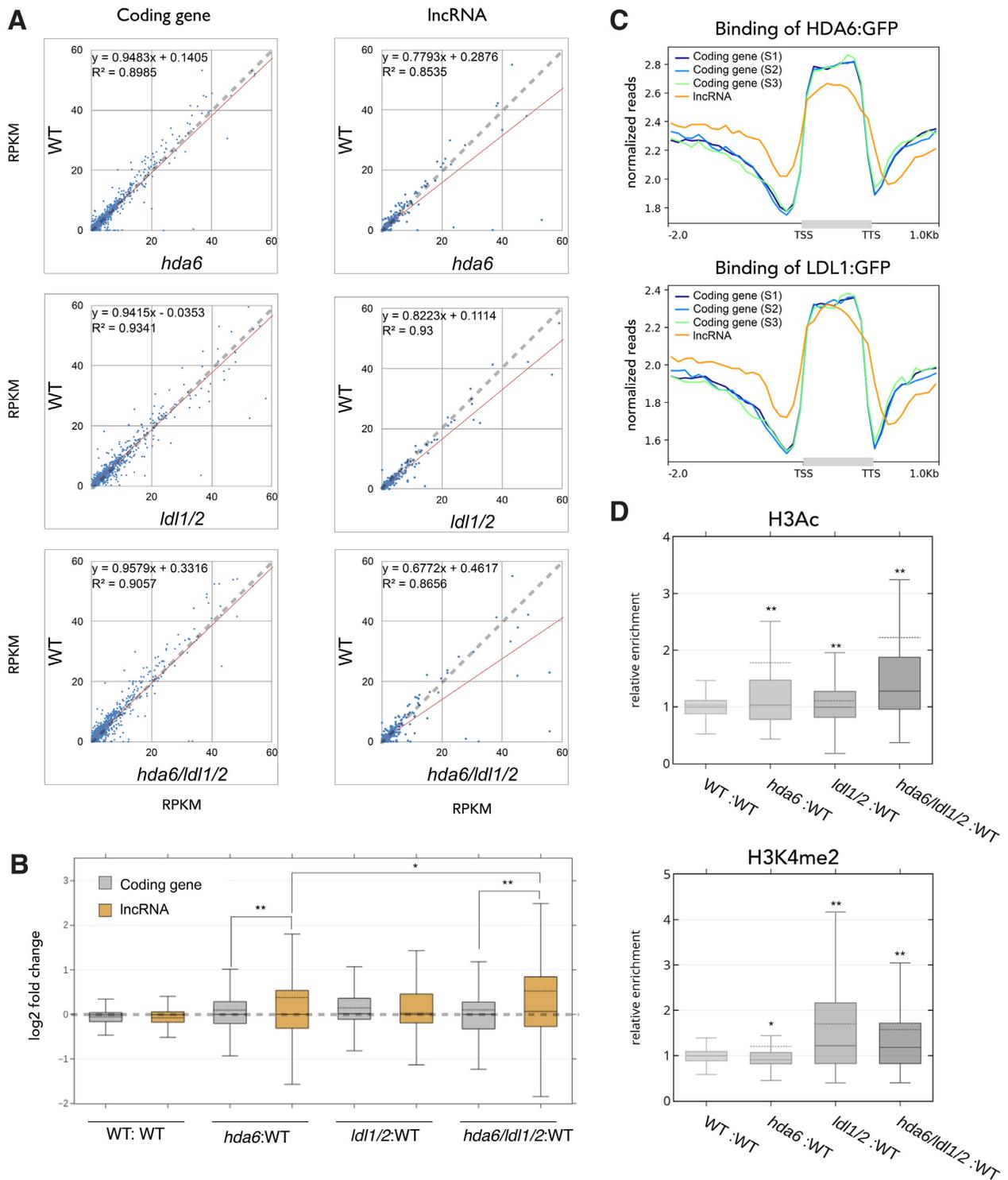
Figure 2. LDL1/2 and HDA6 regulate lncRNA expression by modulating H3K4me2 and H3Ac levels.(**A**) X-Y scatter plot and abline of WT-*hda6*, WT-*ldl1/2* and WT-*hda6/ldl1/2* showing the expression patterns of the protein-coding gene subset and lncRNAs. (**B**) Boxplots showing the relative expression of the protein-coding gene subset and lncRNAs in *hda6*, *ldl1/2* and *hda6/ldl1/2* compared to those in WT. *$P < 0.01$, **$P < 0.001$ (Student's *t*-test). (**C**) Metagene ChIP-seq binding profiles of HDA6 and LDL1 in lncRNAs and the protein-coding gene subsets (S1–S3). The profile is from 2 kb upstream of the TSS to 1 kb downstream of the TTS, and the gene lengths were scaled to the same size. (**D**) Boxplots showing the relative enrichment of H3Ac and H3K4me2 in lncRNAs in *hda6*, *ldl1/2* and *hda6/ldl1/2* compared to those in WT. The dotted line indicates the mean value of each group. *$P < 0.01$, **$P < 0.001$ (Student's t-*test*).
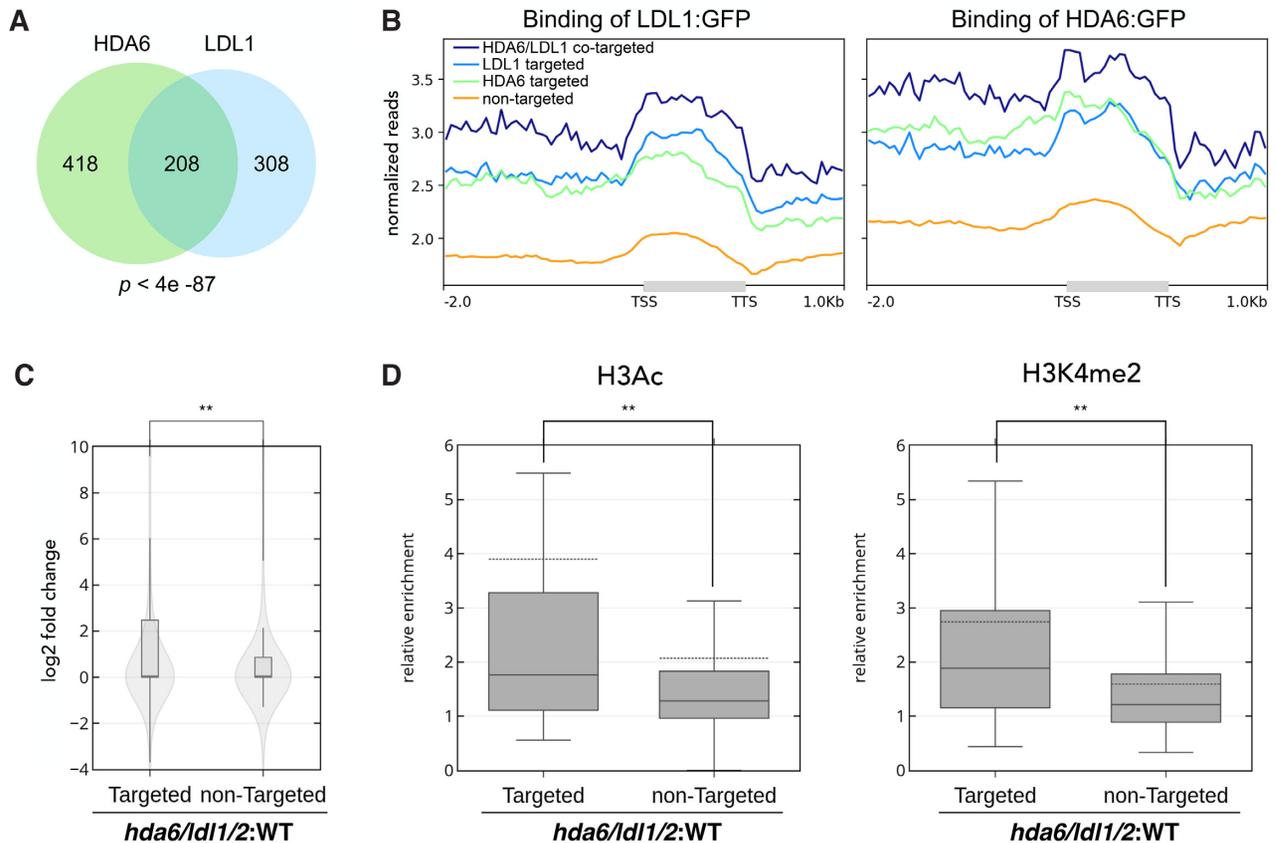
Figure 3. The expression of H3Ac and H3K4me2 in HDA6/LDL1-targeted lncRNAs is increased in *hda6/ldl1/2* compared to WT. (**A**) A Venn diagram showing statistically significant overlap between lncRNAs targeted by HDA6 and those targeted by LDL1 (208 lncRNAs; $P < 4e$ -87, hypergeometric test). (**B**) Metagene ChIP-seq binding profiles of HDA6 and LDL1 in HDA6-targeted lncRNAs, LDL1-targeted lncRNAs, HDA6/LDL1-cotargeted lncRNAs and lncRNAs not targeted by HDA6 or LDL1. The profile is from 2 kb upstream of the TSS to 1 kb downstream of the TTS, and the gene lengths were scaled to the same size. (**C**) Violin plots with boxplots showing the relative expression on the HDA6/LDL1-cotargeted and nontargeted lncRNAs in *hda6/ldl1/2* compared to those in WT. **$P < 0.001$ (Student's *t*-test). (**D**) Boxplots showing the relative enrichment of H3Ac and H3K4me2 in the HDA6/LDL1-cotargeted and non-targeted lncRNAs in *hda6/ldl1/2* compared to those in WT. The dotted line indicates the mean value of each group. **$P < 0.001$ (Student's *t*-test).

that the relative H3Ac and H3K4me2 enrichment levels of the lncRNAs were also increased in *hda6, ldl1/2* and *hda6/ldl1/2* (Figure 2D), indicating that LDL1, LDL2 and HDA6 are involved in the regulation of lncRNAs by affecting H3K4me2 and H3Ac levels.

To determine whether the regulation of lncRNA is associated with other HDACs in *Arabidopsis*, we also analyzed the H3Ac ChIP-seq profiles of WT and *hda9* (64) to compare the changes in H3Ac in lncRNAs. We found that the relative H3Ac level of the lncRNA in *hda9* was not significantly increased compared with that in *hda6* (Supplementary Figure S7A), indicating that lncRNAs are not universally regulated by all HDACs in *Arabidopsis*.

The binding of HDA6 and LDL1 to lncRNAs is associated with increased H3Ac and H3K4me2 levels

A recent study systematically annotated a total of 6510 lncRNAs in *Arabidopsis thaliana* via high-depth strand-specific RNA sequencing (15). These lncRNAs were used for further analysis in this study. We found that H3Ac and H3K4me2 levels in WT *Arabidopsis* were associated with

the distance between neighboring genes, where the lncR-NAs exhibiting higher H3Ac and H3K4me2 levels appeared to be located closer to their neighboring genes (Supplementary Figure S7B). However, we also found that lncRNAs that were located farther from their neighboring genes appeared to be more enriched in *hda6/ldl1/2* (Supplementary Figure S7C). These results suggested that HDA6-LDL1/2 may only regulate the expression of specific lncRNAs.

Among the annotated lncRNAs, 626 and 516 were directly targeted by HDA6 and LDL1, respectively (Figure 3A). A total of 208 lncRNAs were cotargeted by HDA6 and LDL1, indicating high overlap between HDA6- and LDL1-targeted lncRNAs (hypergeometric test, $P < 4e$ -87) (Figure 3A). As we expected, the binding of HDA6 and LDL1 to the targeted lncRNAs was stronger than their binding to non-targeted lncRNAs (Figure 3B). Furthermore, the binding of HDA6 and LDL1 was increased in the HDA6/LDL1-cotargeted lncRNAs (Figure 3B). We further compared the relative expression of the HDA6/LDL1-cotargeted and non-targeted lncRNAs. The relative expression of the HDA6/LDL1-cotargeted lncRNAs in *hda6/ldl1/2* was significantly increased compared to that of the non-targeted
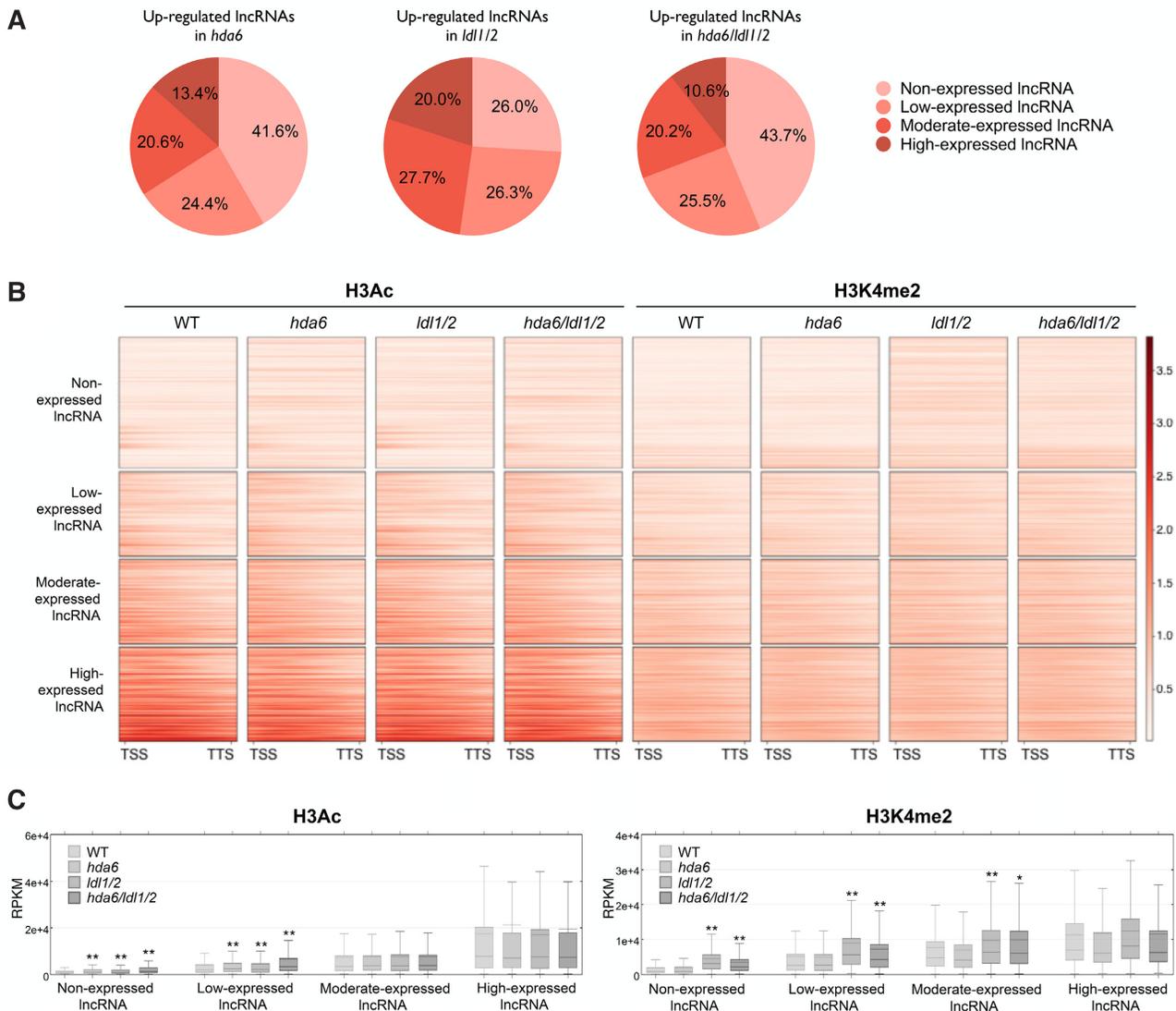
Figure 4. The H3Ac and H3K4me2 levels of lncRNAs that are nonexpressed or show low expression are increased in *hda6/ldl1/2*. (**A**) Pie chart showing the distribution of lncRNAs that were non-expressed or showed low-, moderate- or high- expression among the lncRNAs that were upregulated in *hda6*, *ldl1/2* and *hda6/ldl1/2*. (**B**) H3Ac and H3K4me2 plot heatmap of lncRNAs in WT, *hda6*, *ldl1/2* and *hda6/ldl1/2* arranged by their expression levels in WT. Each horizontal line represents one lncRNA, and the signal intensity is shown for H3Ac or H3K4me2 levels in WT, *hda6*, *ldl1/2* and *hda6/ldl1/2*. Signal intensity is indicated by the shade of red. (**C**) Boxplots showing the H3Ac and H3K4me2 levels of lncRNAs that were non-expressed or showed low-, moderate- or high-expression in WT, *hda6*, *ldl1/2* and *hda6/ldl1/2*. The dotted line indicates the mean value of each group. *$P < 0.05$, **$P < 0.005$ (Student's *t*-test).

lncRNAs (Figure 3C). In addition, the relative H3Ac and H3K4me2 levels of the HDA6/LDL1-cotargeted lncRNAs in *hda6/ldl1/2* were increased compared to those of the non-targeted lncRNAs (Figure 3D). These results indicated that the changes in the H3Ac and H3K4me2 levels of the lncR-NAs are associated with the binding of HDA6 and LDL1.

Among the 6510 annotated lncRNAs, 475, 284 and 526 were upregulated in *hda6*, *ldl1/2* and *hda6/ldl1/2*, respectively (Supplementary Figure S8A). In addition, among the 526 lncRNAs that were upregulated in *hda6/ldl1/2*, 307 and 253 displayed increases in H3Ac (hypergeometric test, $P < 6e-57$) and H3K4me2 (hypergeometric test, $P < 3.7e-20$), respectively, in *hda6/ldl1/2* (Supplementary Figure S8B). We classified the 6510 annotated lncRNAs into non-expressed, low expression, moderate expression and high expression groups according to their expression levels in WT. Interestingly, we found that most of the lncRNAs that were upregulated in *hda6*, *ldl1/2* and *hda6/ldl1/2* were non-expressed or expressed at low levels in WT (Figure 4A and Supplementary Figure S8A). In addition, most of the HDA6/LDL1-targeted lncRNAs were also non-expressed or expressed at low levels (Supplementary Figure S8C and D). The levels of H3Ac and H3K4me2 among the lncRNAs that were non-expressed or showed low expression were lower than those in the highly expressed lncRNAs (Figure 4B and C), indicating that H3Ac and H3K4me2 levels are correlated with the expression levels of lncRNAs. Moreover, we found that the total H3Ac and H3K4me2 levels of the
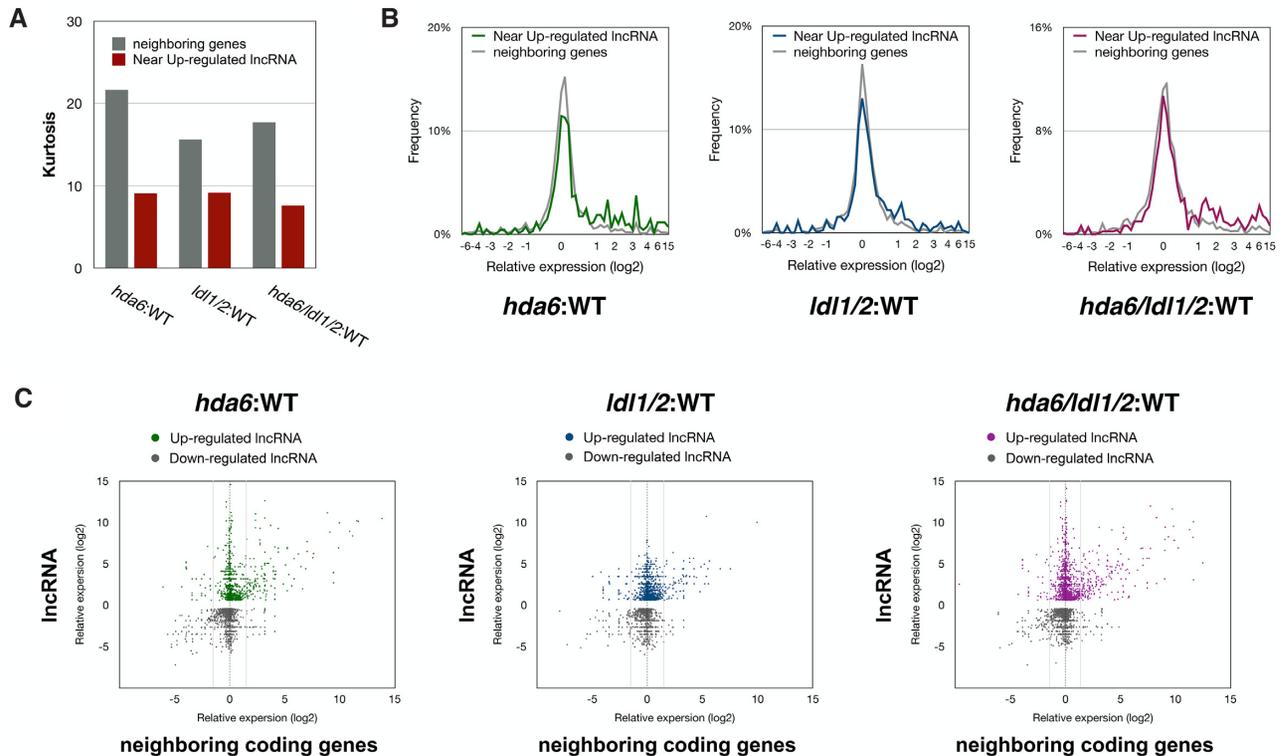
Figure 5. The expression changes in neighboring coding genes located near HDA6/LDL1/2-regulated lncRNAs. (**A**) The statistical kurtosis values of the relative expression levels in WT-*hda6*, WT-*ldl1/2* and WT-*hda6/ldl1/2*. All annotated neighboring coding genes (gray) (15) and the neighboring coding genes located close to the upregulated lncRNAs (red) are presented. (**B**) The distribution of relative expression levels in WT-*hda6*, WT-*ldl1/2* and WT-*hda6/ldl1/2*. Data is shown for all annotated neighboring coding genes (15) and the neighboring coding genes located close to upregulated lncRNAs. (**C**) X-Y scatter plot showing the relative expression levels of the lncRNAs and their neighboring genes in WT-*hda6*, WT-*ldl1/2* and WT-*hda6/ldl1/2*.

lncRNAs that were non-expressed or showed low expression in *hda6*, *ldl1/2* and *hda6/ldl1/2* were increased compared to those in WT (Figure 4B and C). In contrast, the total H3Ac and H3K4me2 levels of the highly expressed lncRNAs in *hda6*, *ldl1/2* and *hda6/ldl1/2* were not changed compared to those in WT (Figure 4B and C). These results suggested that HDA6-LDL1/2 played an important role in the repression of lncRNAs that were nonexpressed or showed low expression. It has been reported that the expression of lncRNAs is highly tissue specific and is regulated by environmental stresses (8–15). Thus, we extracted the ABA-, drought- and cold-induced lncRNAs as well as the lncRNAs showing high expression in inflorescences or siliques identified in a previous study (15) for further analysis. We found that these stress-induced or tissue-specific lncRNAs were mostly nonexpressed or showed low expression under regular treatment (Supplementary Figure S9). Altogether, these results implied that the regulation of lncRNAs that were nonexpressed or showed low expression may be essential for plant development and responses to the environment.

The neighboring genes of the lncRNAs that are upregulated in *hda6/ldl1/2* are associated with development in *Arabidopsis*

Previous studies have revealed that lncRNAs may positively or negatively regulate the expression of their neighboring genes (3–9,15,24–30). The kurtosis value is a measure of

deviation, with lower kurtosis values indicating that there are more extreme values in the sample population. We calculated the kurtosis values of the relative expression levels of the neighboring genes near lncRNAs and found that the kurtosis values of the neighboring genes near the upregulated lncRNAs in *hda6*, *ldl1/2* and *hda6/ldl1/2* were lower than those of the overall neighboring genes (Figure 5A). In addition, the frequencies of extreme values among the neighboring genes near the upregulated lncRNAs in *hda6*, *ldl1/2* and *hda6/ldl1/2* were higher than those among the overall neighboring genes (Figure 5B). When the expression levels of the lncRNAs and their neighboring genes in *hda6*, *ldl1/2* and *hda6/ldl1/2* were compared to those in WT, we found that the expression variation of neighboring genes among the upregulated lncRNAs was higher than that among the downregulated lncRNAs (Figure 5C). These results suggested that the expression levels of neighboring genes are associated with the changes in the expression of lncRNAs.

In addition, we found that the upregulation of lncRNAs in *hda6*, *ldl1/2* and *hda6/ldl1/2* was generally positively correlated with the expression of neighboring genes (Supplementary Figure S10A), which was consistent with the results of a previous study (15). The function of these neighboring genes near the upregulated lncRNAs in *hda6/ldl1/2* was further analyzed according to Gene Ontology Biological Processes (GO-BP). These genes were shown to be involved in various developmental processes, including shoot
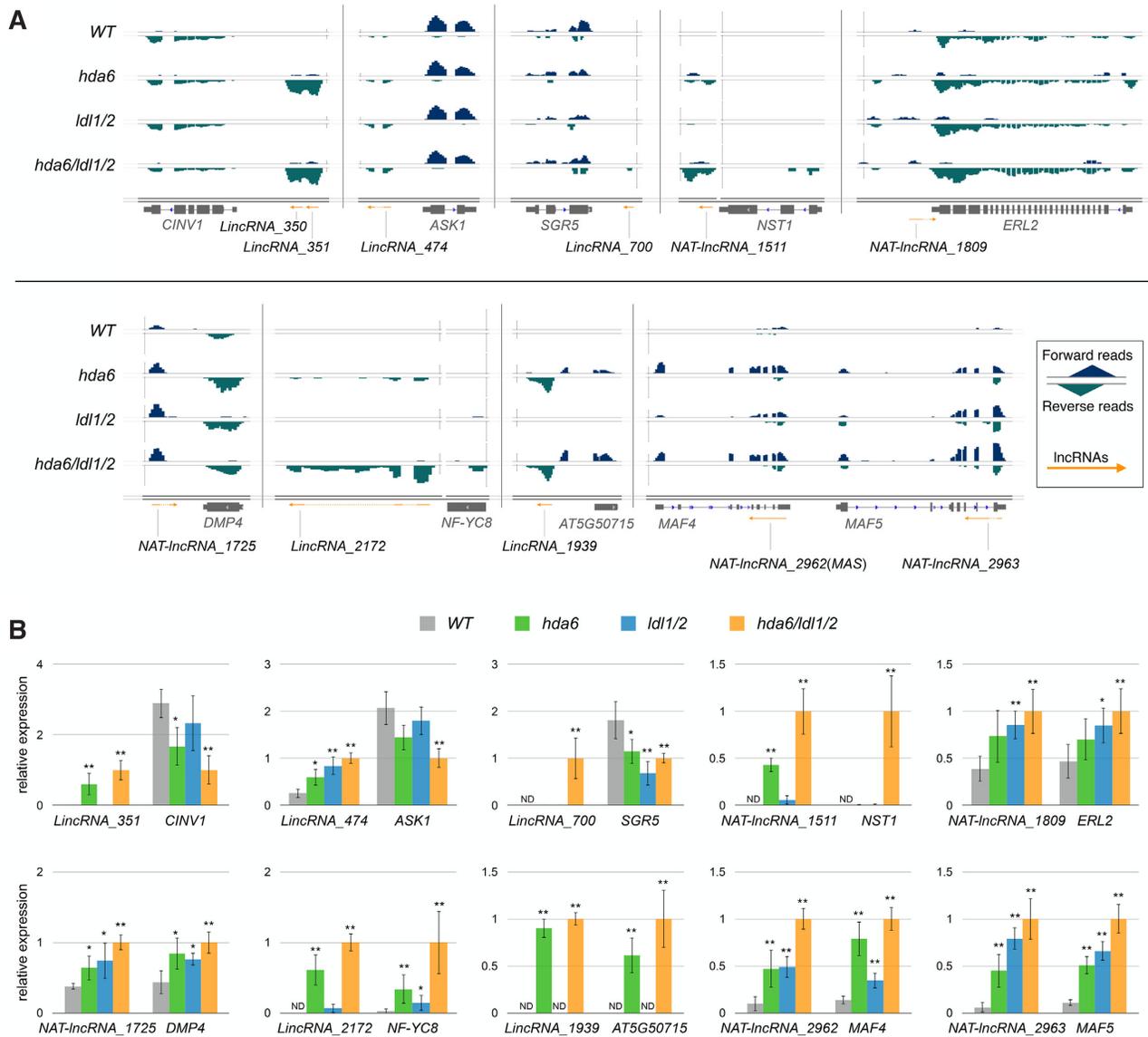
Figure 6. The expression of HDA6-LDL1/2-regulated lncRNAs and their neighboring genes. (**A**) Integrated Genome Viewer showing forward- and reverse-transcript reads of the selected lncRNA genes and their neighboring genes in WT, *hda6*, *ldl1/2* and *hda6/ldl1/2*. Orange arrows indicate transcripts of lncRNA genes from previously published data (15). Bars: normalized reads = 2. (**B**) Gene expression of the selected lncRNA genes and their neighboring genes in WT, *hda6*, *ldl1/2* and *hda6/ldl1/2*. Gene expression was determined by qRT-PCR and normalized to *UBQ10*. N.D.: not detected. Error bars correspond to standard deviations from three biological replicates. *: $P < 0.05$, **: $P < 0.005$ (Student's *t*-test).

and flower development (Supplementary Figure S10B and C). These results suggested that the neighboring genes of the lncRNAs that are upregulated in *hda6/ldl1/2* are associated with various developmental processes in *Arabidopsis*. To further confirm the expression patterns of the lncRNAs showing upregulation in *hda6/ldl1/2* and their neighboring genes, the RNA-seq reads for the forward strand and reverse strand were visualized separately for WT, *hda6*, *ldl1/2* and *hda6/ldl1/2*. We found that the reads of the relevant lncRNAs exhibited substantially higher levels in *hda6/ldl1/2* than in WT (Figure 6A and Supplementary Figure S11). Moreover, we found that the expression of the genes near or overlapping with these lncRNAs was substantially changed in *hda6/ldl1/2* (Figure 6A and Supplementary Figure S11).

We selected 10 lncRNAs and their neighboring genes to further validate their expression by qRT-PCR (Figure 6B). These selected up- or downregulated neighboring genes, including *cytosolic invertase 1* (*CINV1*), *arabidopsis skp1 homologue 1* (*ASK1*), *shoot gravitropism 5* (*SGR5*), *nac secondary wall thickening promoting factor1* (*NST1*), *rrecta-like 2* (*ERL2*), *duf679 domain membrane protein 4* (*DMP4*), *nuclear factor Y subunit C8* (*NF-YC8*), *wrky DNA-binding protein 17* (*WRKY17*), *mads affecting flowering 4* (*MAF4*) and *mads affecting flowering 5* (*MAF5*), are reported to be involved in plant development. ASK1 is a scaffold linker protein of the SCF ubiquitin-protein E3 ligase complex that has been reported to be involved in multiple developmental processes, including early development, flowering time
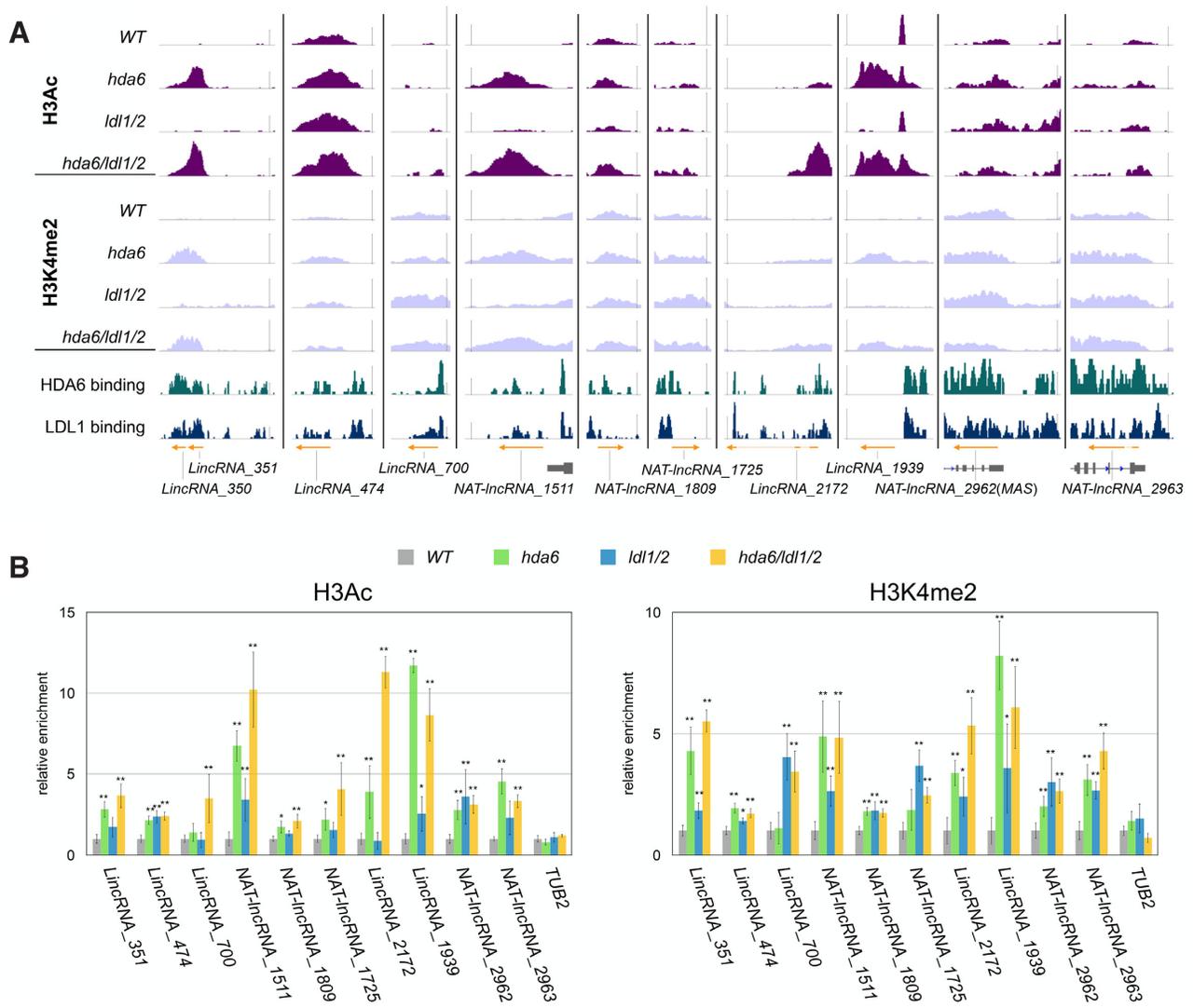
Figure 7. H3Ac and H3K4me2 in the lncRNAs regulated by HDA6-LDL1/2. (**A**) Integrated Genome Viewer showing H3Ac and H3K4me2 peaks in WT, *hda6*, *ldl1/2* and *hda6/ldl1/2* and the binding of HDA6 and LDL1 in the selected lncRNA genes. Orange arrows indicate transcripts of lncRNA genes from previously published data (15). Bars: normalized reads = 5. (**B**) ChIP analysis of H3Ac and H3K4me2 levels of the selected lncRNA genes. ChIP assays were performed with H3K9K14Ac and H3K4me2 antibodies. The relative enrichment was determined by qRT-PCR and normalized to *ACT2*. Error bars correspond to standard deviations from three biological replicates. *: $P < 0.05$, **: $P < 0.005$ (Student's *t*-test).

and the circadian clock (65–68). *SGR5* is highly expressed in petioles, hypocotyls, roots, floral organs and especially inflorescence stems and is involved in shoot and flower development (69,70). ERL2 is associated with organ growth and flower development by promoting cell proliferation (71). NF-YC8 is a subunit of the NF-Y DNA-binding complex, and *Arabidopsis* NF-YC homologs act as important factors in flowering time control (72,73). In addition, NST1 and DMP4 have been reported to be involved in flower development (74,75), whereas CINV1, ASK1 and WRKY17 have been reported to be involved in root development and abiotic stress responses in *Arabidopsis* (76–78).

MAF4 and MAF5 are paralogs of FLC and act as floral repressors in *Arabidopsis* (79). *MAS* (*NAT-lncRNA_2962*) is an antisense lncRNA produced from the 3′ region of *MAF4* that acts as a positive regulator of *MAF4* (15). In our study, we found that the expression of *MAS* was increased in *hda6*,

ldl1/2 and *hda6/ldl1/2* (Figure 6A and B). *MAF4* expression was also increased in *hda6*, *ldl1/2* and *hda6/ldl1/2* (Figure 6A and B). Similar to *MAS*, *NAT-lncRNA_2963* is an antisense lncRNA transcribed from the 3′ region of *MAF5* (15). The expression of both *NAT-lncRNA_2963* and *MAF5* was increased in *hda6*, *ldl1/2* and *hda6/ldl1/2* (Figure 6A and B).

The HDA6/LDL1 binding peaks and H3Ac and H3K4me2 profiles of these lncRNAs in WT, *hda6*, *ldl1/2* and *hda6/ldl1/2* were viewed and curated in IGV. We identified specific binding of HDA6 and LDL1 to the promoter regions of these lncRNAs (Figure 7A; Supplementary Figures S12 and 13). Moreover, H3Ac and H3K4me2 levels were substantially increased in the downstream regions of HDA6 and LDL1 binding sites in *hda6/ldl1/2*, and the increased H3Ac and H3K4me2 peaks were located near the transcribed regions of these lncRNAs (Figure 7A; Supple-

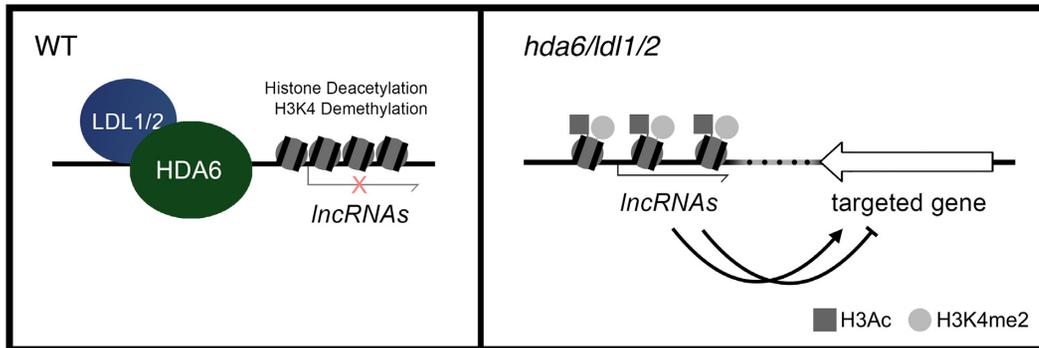## non- and low- expressed lncRNAs

Figure 8. The expression of lncRNAs that are nonexpressed or show low expression is associated with H3Ac/H3K4me2 changes regulated by the HDA6-LDL1/2 histone modification complex. The HDA6-LDL1/2 histone modification complex can bind to the promoter region or gene body of lncRNAs that are non-expressed or show low expression to suppress their expression by histone deacetylation and H3K4 demethylation. The expression of protein-coding genes may be positively or negatively regulated by their neighboring lncRNAs; therefore, HDA6-LDL1/2 may affect the expression of protein-coding genes by regulating neighboring lncRNAs.

mentary Figure S12 and 13). The H3Ac and H3K4me2 levels of these lncRNAs were further validated by ChIP–qPCR (Figure 7B). It appears that HDA6 and LDL1 can bind to the promoters of specific lncRNAs to regulate their expression. Furthermore, we extracted all the neighboring genes of the lncRNAs that were upregulated in *hda6/ldl1/2*. The general binding of HDA6 and LDL1 to the promoters of these neighboring genes was reduced compared with that among all genes in *Arabidopsis* (Supplementary Figure S14), suggesting that these neighboring genes may not be directly regulated by HDA6 and LDL1.

Among the neighboring genes located near the lncRNAs that were upregulated in *hda6/ldl1/2*, over 75% were protein-coding genes, whereas ∼19% were transposable element (TE) genes (Supplementary Table S2). We found that the neighboring genes of the lncRNAs that were upregulated in *hda6/ldl1/2* may be associated with various developmental processes in *Arabidopsis*. Both *hda6* and *ldl1/2* mutants are late flowering (45,48). Interestingly, we found that *hda6/ldl1/2* plants flowered later than *hda6* and *ldl1/2* plants (Supplementary Figure S15A and B). The enhanced late-flowering phenotype of *hda6/ldl1/2* may be associated with the lncRNA-neighboring genes regulated by HDA6-LDL1/2.

DISCUSSION

HDACs and LSD1 have been identified as the core components of several multiprotein complexes, such as Mi2/NuRD and CoREST, that regulate gene expression cooperatively in yeast and animal systems (32–36). It has also been found that human HDAC1/2 and LSD1 are aberrantly highly expressed and positively regulate cell growth in many kinds of cancer cell lines (80–82), suggesting that the HDAC complex is important in cell growth and development. In this study, we found that HDA6 and LDL1 are associated with promoter and gene body regions. A previous global binding analysis of human HDACs also showed that HDAC2 and HDAC6 can bind to both promoter and gene body regions (83). The binding of human HDACs is

associated with the function of RNA Pol II and the repression of transcription elongation in human cells (84–86). A recent study revealed that human HDACs are correlated with the positive regulation of transcription elongation (86). Furthermore, the binding of human HDACs to gene bodies is involved in the regulation of alternative splicing (87–90). The binding of *Arabidopsis* HDA6 and LDL1 in gene body regions implies that the HDA6-LDL1/2 complex may also be involved in transcription elongation and alternative splicing. In this study, we found that HDA6-LDL1/2 is associated with the regulation of lncRNAs. Because ∼30% of the annotated lncRNAs are antisense transcripts and overlap with protein-coding genes (15), it is possible that the gene body regions bound by HDA6 and LDL1 may act as the promoters of the overlapping lncRNAs.

In plants, lncRNAs have been implicated in development, light responses, vernalization, auxin transport and stress responses (15–28). However, only a few plant lncRNAs have been studied in detail. In addition, the expression of lncRNAs is lower than that of protein-coding genes, and they tend to be more tissue specific (8–12). Furthermore, the expression of plant lncRNAs is lower than that of animal lncRNAs (10,15). Recently, it was reported that the expression of the human lncRNAs *TINCR* and *PLAC2* is regulated by histone acetylation (91,92). However, the mechanism of lncRNA expression regulation in plants is still largely unclear. In this study, we found that a subset of *Arabidopsis* lncRNAs that are nonexpressed or show low expression are regulated by HDA6-LDL1/2. Because the expression of lncRNAs is highly tissue specific and can be induced under certain environmental conditions (1,10,13–15), HDA6-LDL1/2 may play an important role in plant development by repressing lncRNAs.

lncRNAs can positively or negatively regulate the expression of their neighboring genes (3–9,15,24–30). We found that a subset of genes located near the HDA6-LDA1/2-regulated lncRNAs are involved in various plant developmental processes. These genes include *CINV1*, *ASK1*, *SGR5*, *NST1*, *ERL2*, *NF-YC8*, *WRKY17*, *MAF4* and *MAF5* (60–74). The misexpression of these neighboring

genes may result in developmental defects in *Arabidopsis*. Previously, HDA6 was reported to repress the expression of *MAF4* and *MAF5* (45). The repression of *MAF4* and *MAF5* by HDA6 may be related to their nearby lncRNAs. Furthermore, HDA6 is involved in the regulation of TEs by interacting with DNA Methyltransferase1 (MET1) and Kryptonite/Suvh4 (KYP) (39–41). Interestingly, we found that ∼19% of the neighboring genes near the upregulated lncRNAs in *hda6/ldl1/2* are TEs. It has been reported that TEs contribute to the tissue-specific and stress-induced expression of lncRNAs (93,94). Therefore, these TEs regulated by HDA6 may also be involved in the regulation of lncRNAs.

Collectively, our research revealed that the expression of lncRNAs that are non-expressed or show low expression is associated with H3Ac/H3K4me2 changes regulated by the HDA6-LDL1/2 histone modification complex (Figure 8). Furthermore, we found that a subset of the neighboring genes of the lncRNAs regulated by HDA6 and LDL1 are associated with various plant developmental processes. These results indicated that the expression of *Arabidopsis* lncRNAs can be suppressed by the HDA6-LDL1/2 complex and that HDA6-LDL1/2 may be associated with plant developmental processes by modulating the expression of lncRNAs.

DATA AVAILABILITY

The RNA-seq and ChIP-seq short read data have been submitted to the NCBI Gene Expression Omnibus (GEO) database, including the previously published LDL1 ChIP-seq data (GSE118025) (46) and unpublished data from HDA6 ChIP-seq (GSE132563), H3Ac ChIP-seq of WT, *hda6*, *ldl1/2* and *hda6/ldl1/2* (GSE132636), H3K4me2 ChIP-seq of WT, *hda6*, *ldl1/2* and *hda6/ldl1/2* (GSE133005) and RNA-seq of WT, *hda6*, *ldl1/2* and *hda6/ldl1/2* (GSE132873).

SUPPLEMENTARY DATA

Supplementary Data are available at NARGAB Online.

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Conflict of interest statement. None declared.

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