Histone Deacetylase HDA6 Is Functionally Associated with AS1 in Repression of KNOX Genes in Arabidopsis

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Abstract

ASYMMETRIC LEAVES 1 (AS1) is a MYB-type transcription repressor that controls leaf development by regulating KNOX gene expression, but the underlying molecular mechanism is still unclear. In this study, we demonstrated that AS1 can interact with the histone deacetylase HDA6 in vitro and in vivo. The KNOX genes were up-regulated and hyperacetylated in the hda6 mutant, axe1-5, indicating that HDA6 may regulate KNOX expression through histone deacetylation. Compared with the single mutants, the as1-1/axe1-5 and as2-1/axe1-5 double mutants displayed more severe serrated leaf and short petiole phenotypes. In addition, the frequencies of leaf lobes and leaflet-like structures were also increased in as1-1/axe1-5 and as2-1/axe1-5 double mutants, suggesting that HDA6 acts together with AS1 and AS2 in regulating leaf development. Chromatin immunoprecipitation assays revealed that HDA6 and AS1 bound directly to KNAT1, KNAT2, and KNATM chromatin. Taken together, these data indicate that HDA6 is a part of the AS1 repressor complex to regulate the KNOX expression in leaf development.

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Introduction

The initiation of leaf primordia is established by recruitment of cells from the flanks of the shoot apical meristem (SAM). Meristem activity in the shoot apex is specified in part by the class I KNOTTED-LIKE HOMOBOX (KNOX) genes [1–3]. Lateral organs, such as leaves, are initiated on the flanks of SAM, and down-regulation of KNOX genes is essential to facilitate this process [1,4]. Moreover, the silencing of KNOX genes is important in developing organs since the ectopic KNOX expression during organogenesis resulted in patterning defects and over-proliferation of cells [5–7]. Thus, the balance between stem cell differentiation and proliferation that is decisive for plant development is attained, in part through the proper regulation of the KNOX expression.

In Arabidopsis, the KNOX family can be further divided into three classes. Class I KNOX genes are similar to KNOTTED1 (KN1) in maize, including BREVIPEDICELLUS (BP)/KNAT1, KNAT2, KNAT6 and SHOOTMERISTEMLESS (STM). These genes are expressed in the SAM and down-regulated in leaf primordia [8]. Class II KNOX genes comprise KNAT3, KNAT4, KNAT5 and KNAT7, which are broadly expressed. Class III only contains KNATM, which is a novel KNOX gene lacking the homeodomain. It was demonstrated that KNATM functions together with KNAT1 and BELL proteins by forming heterodimer [9]. Moreover, ectopic expression of KNATM resulted in the curled down and serrated rosette leaves in wild type plants [9]. KNOX repression is mediated by the orthologous MYB domain proteins ROUGH SHEATH2 (RS2) in maize (Zea mays) and ASYMMETRIC LEAVES1 (AS1) in Arabidopsis thaliana [10–13]. In addition, AS1 interacts with the LATERAL ORGAN BOUNDARIES (LOB) domain protein AS2 and directly represses the expression of BP/KNAT1 and KNAT2 [14–16]. Previous studies revealed that AS1 and AS2 may recruit a chromatin-remodeling protein Histone Regulatory Homolog 1 (HIRA) to regulate the expression of target genes [17]. Moreover, HIRA has also been shown to interact with a histone deacetylase (HDAC) in animal cells [18].

In this study, we investigated the interaction of AS1 with the histone deacetylase HDA6 and their involvement in leaf development. We demonstrated that HDA6 can interact with AS1 in vivo and in vitro. The hda6 mutant, axe1-5, displayed curling and serrated leaves as well as shorter petioles, suggesting that HDA6 is involved in leaf development. Additionally, HDA6 and AS1 associate directly with the promoters of KNAT1, KNAT2 and KNATM. Taken together, our data suggest that HDA6 is a part of the AS1 repression complex to regulate the expression of KNOX genes.
Author Summary

AS1 is a MYB-type transcription repressor that controls leaf patterning by repressing class-I KNOX gene expression. The molecular mechanism by which AS1 represses KNOX gene expression is still unclear. In yeast and mammalian cells, many transcription repressors were found to recruit HDACs to regulate their target genes [19]. To further understand the molecular mechanism of AS1-dependent KNOX repression, we analyzed the interaction of AS1 with HDA6, a RPD3-type HDAC in Arabidopsis [20,21] by using BiFC assays. The coding sequences of HDA6 and AS1 were fused to the N-terminal 174-amino acid portion of yellow fluorescent protein (YFP) in the pEarley-Gate201 vector (pEarleyGate201-YN) or the C-terminal 66-amino acid portion of YFP in the pEarleyGate202 vector (pEarleyGate202-YC) [22]. The Agrobacterium cells containing these constructs were co-transfected into Nicotiana benthamiana leaves. The yellow fluorescence was observed at the nuclear when HDA6-YN and AS1-YC were transient expressed in N. benthamiana leaves, indicating that HDA6 interacted with AS1 in vivo (Figure 1A). In contrast, the yellow fluorescence was not observed in the negative controls (Figure S1).

The interaction between HDA6 and AS1 was further confirmed by in vitro pull down assays. When purified MBP-AS1 recombinant protein was incubated with glutathione S-transferase (GST)-HDA6 protein, HDA6-GST was pulled down by MBP-AS1 (Figure 1B), indicating that HDA6 was directly associated with AS1. Co-immunoprecipitation (CoIP) assays were also used to analyze the interaction between HDA6 and AS1. A stable transgenic plant expressing 35S:GFP-HDA6 in the hda6 mutant (axe1-5) was generated [23]. Overexpressing 35S:GFP-HDA6 in axe1-5 complemented the mutant phenotype, suggesting that the GFP-HDA6 fusion protein is functional. Crude extracts (input) of axe1-5, ax1-1 and axe1-5/35S:GFP-HDA6 were immunoprecipitated by the AS1 antibody, then analyzed by western blotting. As shown in Figure 1C, GFP-HDA6 was clearly co-immunoprecipitated by endogenous AS1. Furthermore, AS1 protein was also co-immunoprecipitated by GFP-HDA6 when immunoprecipitated by the GFP antibody (Figure 1C). Taken together, our data strongly indicate that HDA6 interacts with AS1 in vitro and in vivo.

AS1 and AS2 can interact and form homo- and hetero-dimers

Previous studies indicated that AS1 and AS2 can associate together both in yeast cells by yeast two-hybrid assays and in vitro by ELISA experiments using purified His-AS1 and GST-AS2 recombinant proteins [14]. By using BiFC assays, we also found that AS1 and AS2 can interact with each other in N. benthamiana leaves (Figure S2). Furthermore, both AS1 and AS2 can also interact with itself (Figure 2A). These observations indicated that AS1 and AS2 can form both homo- and hetero-dimers. The yellow fluorescence was observed at the nucleus when AS1-YN and AS1-YC, AS2-YN and AS2-YC, or AS1-YN and AS2-YC were transient expressed in N. benthamiana leaves (Figure S2). Moreover, the in vivo interaction between HDA6 and AS2 was also found by using BiFC (Figure 2B). Collectively, these results together with the finding that HDA6 interacts with AS1 suggested that HDA6, AS1 and AS2 function together in the same protein complex.

We further tested the protein-protein interactions among HDA6, AS1 and AS2 in the proplasts isolated from the mutants. By using BiFC assays, we found that HDA6 interacted with AS1 in

Figure 1. HDA6 interacted with AS1. (A) BiFC in N. benthamiana leaves showing interaction between HDA6 and AS1 in living cells. HDA6 and AS1 fused with the N terminus (YN) or C terminus (YC) of YFP were codelivered into tobacco leaves by Agrobacterium GV3101 and visualized using a confocal microscope. The nucleus was stained with Hoechst staining (Blue). Bars = 200 µm. (B) HDA6 interacted with AS1 in Arabidopsis. Crude extracts of transgenic plants expressing 35S:GFP-HDA6 in axe1-5 background were immunoprecipitated (IP) with AS1 or GFP antibody and analyzed by western blotting. axe1-1 and axe1-5 mutant plants were used as the negative control.

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Results

HDA6 interacts with AS1 in vitro and in vivo

AS1 is a MYB-type transcription repressor that controls leaf patterning by repressing class-I KNOX gene expression [16]. However, the molecular mechanism how AS1 represses KNOX gene expression is still unclear. In yeast and mammalian cells, many transcription repressors were found to recruit HDACs to regulate their target genes [19]. To further understand the molecular mechanism of AS1-dependent KNOX repression, we analyzed the interaction of AS1 with HDA6, a RPD3-type HDAC in Arabidopsis [20,21] by using BiFC assays. The coding sequences of HDA6 and AS1 were fused to the N-terminal 174-amino acid portion of yellow fluorescent protein (YFP) in the pEarley-Gate201 vector (pEarleyGate201-YN) or the C-terminal 66-amino acid portion of YFP in the pEarleyGate202 vector (pEarleyGate202-YC) [22]. The Agrobacterium cells containing these constructs were co-transfected into Nicotiana benthamiana leaves. The yellow fluorescence was observed at the nuclear when HDA6-YN and AS1-YC were transient expressed in N. benthamiana leaves, indicating that HDA6 interacted with AS1 in vivo (Figure 1A). In contrast, the yellow fluorescence was not observed in the negative controls (Figure S1).

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We further tested the protein-protein interactions among HDA6, AS1 and AS2 in the proplasts isolated from the mutants. By using BiFC assays, we found that HDA6 interacted with AS1 in
the petioles were decreased in *axe1-5* mutants (Figure 3D). *as1-1/axe1-5* and *as2-1/axe1-5* double mutants displayed shorter petioles compared with *as1-1* and *as2-1* single mutant plants (Figure 3B, 3C and Figure 3D). However, the lamina lengths of *as1-1/axe1-5* and *as2-1/axe1-5* did not show significant changes compared with the single mutants (Figure 3E).

We further measured the frequencies of leaf lobe formation in *axe1-5, sil1, as1-1/axe1-5* and *as2-1/axe1-5* mutants. The frequencies of leaf lobes were significantly increased in *as1-1/axe1-5* and *as2-1/axe1-5* double mutants (Table 1). *as2* mutants produced leaflet-like structures on the petioles [26]. In *as1-1/axe1-5* and *as2-1/axe1-5* double mutants, the frequencies of leaflet-like structures were increased (Table 2), and some of the leaf lobes were similar to leaflet-like structures (Figure 3C). These results suggested that *HDA6* acts with *AS1* and *AS2* in regulating leaf development.

### Expression of KNOX genes was increased in *axe1-5, as1-1*, and *as2-1* mutant plants

We further analyzed the gene expression by quantitative reverse transcription (qRT)-PCR in mutant plants. Compared with Col wild type, no significant changes were found in the expression of *AS1* and *AS2* in the *axe1-5* (Figure S5). As shown in Figure 4, the expression of *KNAT1, KNAT2* and *KNATM* was increased in *axe1-5* compared to Col wild type. Consistent with the previous study [13], the transcript levels of *KNAT1* and *KNAT2* were elevated in *as1-1* and *as2-1* mutant plants. In addition, the expression of *KNATM* was also up-regulated in *as1-1* and *as2-1* mutant plants. Moreover, the expression of *KNAT1, KNAT2* and *KNATM* was highly increased in *as1-1/axe1-5* and *as2-1/axe1-5* double mutants compared with their corresponding single mutants. These data indicate that *HDA6* may function synergistically with *AS1* and *AS2* in regulating the expression of KNOX genes.

We also analyzed the expression of *PHB, PHV, CUC1* and *CUC2*, which were involved in leaf development through the miRNA regulated pathway [27–30]. However, no significant different was found in the expression of *PHB, PHV, CUC1* and *CUC2* (Figure S5).

### Histone H3 acetylation levels of KNOX genes were increased in *axe1-5, as1-1/axe1-5*, and *as2-1/axe1-5* mutant plants

To determine whether the high expression of KNOX genes in the mutants is related to histone hyperacetylation in chromatin, ChIP assays were used to analyze the histone H3 acetylation levels of *KNAT1, KNAT2* and *KNATM*. The relative enrichment of histone H3 acetylation were slightly elevated in the proximal promoter and transcription start regions of individual genes. As shown in Figure 5, levels of histone H3 acetylation were almost similar in the proximal promoter and transcription start regions of *KNAT1, KNAT2* and *KNATM* in *axe1-5*, suggesting that *HDA6* may regulate these genes expression by chromatin deacetylation. We further analyzed histone acetylation levels of *KNAT1, KNAT2* and *KNATM* in *as1-1, as2-1* and the double mutants. As shown in Figure 5B, hyperacetylation of histone H3 was found in the promoter and first exon of *KNAT1, KNAT2* and *KNATM* in *as1-1/axe1-5* and *as2-1/axe1-5* double mutants. In contrast, hyperacetylation of histone H3 was not found in *as1-1* and *as2-1* single mutants. These results suggested that hyperacetylation of histone H3 in *KNAT1, KNAT2* and *KNATM* found in *as1-1/axe1-5* and *as2-1/axe1-5* double mutants was caused by the *hda6* mutation.
Figure 3. Phenotypes of axe1-5, sil1, as1-1, as2-1, as1-1/axe1-5, and as2-1/axe1-5. (A) The margin serration and curling leaf phenotype of hda6 mutants. Col, axe1-5 and sil1 plants were grown for 25 days under LD conditions or 75 days under SD conditions. (B) as1-1/axe1-5 and as2-1/axe1-5 double mutants displayed a more severe curling and serration leaf phenotype. (C) The phenotype of leaf lobes and leaflet-like structures in axe1-5, as1-1, as2-1, as1-1/axe1-5 and as2-1/axe1-5. Arrows indicate the leaf lobes in axe1-5 mutants; or leaflet-like structures in as1-1/axe1-5 and as2-1/axe1-5 double mutants. (D) Quantification of the petiole lengths of 5th leaves to 11th leaves. (E) Quantification of the lamina lengths of 5th leaves to 11th leaves. Plants grown under LD conditions for 32 days were used to measure the petiole and lamina lengths. Error bars show SD (n>12). doi:10.1371/journal.pgen.1003114.g003
Histone H3K4Me3 is another chromatin mark associated with active genes. We also investigated the histone H3K4Me3 level in as1-1 mutants. However, no significant changes in the H3K4Me3 of KNAT1, KNAT2, and KNATM were found (Figure S6A). H3K9Me2 was reported as a chromatin marker associated with gene repression. No significant changes in the level of histone H3K9Me2 were found in as1-1 mutants (Figure S6B).

HDA6 and AS1 bound to KNAT1, KNAT2, and KNATM chromatin

The direct association between AS1 and HDA6 suggested that AS1 may recruit HDA6 to repress the downstream target genes. Previous studies demonstrated that the AS1 repressor complex binds directly to the regulatory motif I (CWGTTD) and motif II (KMKTGGAHW) on the promoters of KNAT1 and KNAT2 [16]. We also found the conserved motif I and motif II in two promoter regions (KNAMT-X and KNAMT-Y) of KNATM (Figure 6A and Figure S7). To investigate whether AS1 binds directly to KNAT1, KNAT2, and KNATM, ChIP analyses using the AS1 antibody were performed in Col wild type and as2-1 mutants. We found the loss of binding of AS1 to the promoters of KNAT1, KNAT2, and KNATM in mutants (Figure 6C), suggesting that AS1 is required to recruit HDA6.

To analyze whether HDA6 is bound directly to KNAT1, KNAT2, and KNATM, transgenic plants expressing HDA6-Myc were subjected to ChIP analysis using an anti-Myc antibody. As shown in Figure 6D, ChIP analyses revealed that HDA6 can bind to the promoters of KNAT1, KNAT2, and KNATM. We also analyze whether HDA6 recruitment is dependent on AS1. ChIP assays were performed using an anti-Myc antibody in transgenic plants expressing the HDA6-Myc in as1 mutants. As shown in Figure 6D, HDA6 cannot bind to KNAT1, KNAT2, and KNATM in as1 mutants, suggesting that AS1 is required to recruit HDA6.

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To analyze whether HDA6 binding is dependent on its catalytic activity, we performed ChIP assays using an anti-FLAG antibody in transgenic plants (HDA6 all 5 mut in axe1-5) expressing the FLAG-tagged HDA6 bearing the five amino acid mutation of the active site in as1-5 mutants [31]. As shown in Figure 6E, the active site mutant HDA6 can still bind to KNAT1, KNAT2, and KNATM, suggesting that HDA6 recruitment is independent of its catalytic activity. Taken together, our findings suggested that HDA6, AS1 and AS2 act together and directly repress the expression of KNOX genes in Arabidopsis.

Discussion

HDA6 regulates the KNOX expression

The Arabidopsis genome sequence contains 9 KNOX genes, which can be further classified into 3 classes [32]. In leaves, AS1 and AS2 down-regulate class I KNOX genes, but not STM; conversely, STM represses AS1 expression in the SAM [12,33]. Downregulation of KNOX genes expression is a vital step in leaf initiation, and silencing of these genes needs to be maintained for normal

| Table 1. Frequency of leaf lobes. |
|----------------------------------|
| Leaf number                     |
| n°                              |
| 1                               |
| 2                               |
| 3                               |
| 4                               |
| 5                               |
| 6                               |
| 7                               |
| 8                               |
| 9                               |
| as1-1                           |
| 8                               |
| 0(0)                            |
| 0(0)                            |
| 0(0)                            |
| 0(0)                            |
| 0(0)                            |
| 0(0)                            |
| 0(0)                            |
| as1-1/axe1-5                    |
| 11                              |
| 10(91)                          |
| 11(100)                         |
| 11(100)                         |
| 11(100)                         |
| 11(100)                         |
| 11(100)                         |
| as1-1/axe1-5                    |
| 12                              |
| 7(58)                           |
| 11(92)                          |
| 10(83)                          |
| 11(92)                          |
| 12(100)                         |
| 12(100)                         |
| 12(100)                         |

Leaves from 35-day-old plants were examined.

| Table 2. Frequency of leaflet-like structure. |
|----------------------------------------------|
| Leaf number                                  |
| n°                                           |
| 1                                            |
| 2                                            |
| 3                                            |
| 4                                            |
| 5                                            |
| 6                                            |
| 7                                            |
| 8                                            |
| 9                                            |
| as1-1                                        |
| 8                                            |
| 0(0)                                        |
| 0(0)                                        |
| 0(0)                                        |
| 0(0)                                        |
| 0(0)                                        |
| 0(0)                                        |
| 0(0)                                        |
| as1-1/axe1-5                                 |
| 11                                          |
| 0(0)                                        |
| 0(0)                                        |
| 0(0)                                        |
| 1(8)                                        |
| 5(46)                                       |
| 4(36)                                       |
| 3(27)                                       |
| 2(18)                                       |
| 8(73)                                       |
| as2-1                                        |
| 8                                            |
| 0(0)                                        |
| 1(13)                                       |
| 0(0)                                        |
| 2(25)                                       |
| 2(25)                                       |
| 2(25)                                       |
| 0(0)                                        |
| 0(0)                                        |
| 4(50)                                       |

Leaves from 35-day-old plants were examined.

Numbers in parentheses show the percentages of leaves on which the leaflet-like structure was observed.

Numbers in parentheses show the percentages of leaves on which a lobe was observed.

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Numbers in parentheses show the percentages of leaves on which the leaflet-like structure was observed.

doi:10.1371/journal.pgen.1003114.t002
PCR analysis of gene expression of KNAT1, KNAT2 and KNATM in as1-1, as2-1, as1-1/axe1-5 and as2-1/axe1-5 double mutants. qRT-PCR analysis of gene expression of KNAT1, KNAT2 and KNATM in as1-1, as2-1, as1-1/axe1-5 and as2-1/axe1-5 double mutants compared to their corresponding single mutants. In addition, levels of histone H3 acetylation was elevated in KNAT1, KNAT2 and KNATM loci in as1-1/axe1-5 and as2-1/axe1-5 double mutants, suggesting that HDA6 is required for the repression of KNOX genes by chromatin deacetylation. ChIP analyses revealed that HDA6 and AS1 bound directly to the promoters of KNAT1, KNAT2 and KNATM. These data indicate that HDA6 and AS1 function together in controlling KNOX gene expression through histone deacetylation. In addition, AS1 is required to recruit HDA6 in KNOX repression.

HDA6 Interacts with AS1

**KNATM is a novel target of the AS1–AS2 complex**

AS1 is a Myb domain transcription factor related to RSI in maize and PHANTASTICA in Antirrhinum [12]. Mutations in AS1 result in abnormal leaves, with marginal outgrowths or lobes [12, 13, 33, 35]. AS2 encodes a LOB domain protein containing a leucine-zipper motif [36–38]. Mutations in the as2 gene cause a phenotype similar to as1 mutants [13, 33]. Previous studies indicated that AS1 and AS2 can associate together both in vitro and in yeast cells [14]. By using the BiFC assay, we found that AS1 and AS2 can interact and form homo and hetero-dimer in plant cells. These data suggested that AS1 and AS2 function in the same protein complex.

A recent study indicated that AS1 functions as a transcriptional repressor and binds directly to its KNOX targets when in a complex with AS2 [16]. It was found that the AS1–AS2 repressor complex binds directly to the regulatory motif I (CWGTTD) and motif II (KMKTTGAHW) in the promoters of the KNAT1, KNAT2 and KNATM [16]. Similar to KNAT1 and KNAT2, we also found the conserved motif I and motif II in the promoter of KNATM. KNATM is a novel Arabidopsis Class III KNOX gene that has a MEINOX domain but lacks the homeodomain [9]. ChIP assays revealed that AS1 can bind directly to the promoter regions of KNAT1, KNAT2 and KNATM. These data suggested that in addition to KNAT1 and KNAT2, the AS1–AS2 complex is also targeted to KNATM by binding to the conserved motifs I and II. To our knowledge, this is the first study demonstrating that KNATM is regulated by AS1 and AS2.

HDA6 is one of the epigenetic components involved in the AS1–AS2–mediated KNOX repression

Recent studies suggested that the AS1–AS2 complex binds to the KNAT1 and KNAT2 promoters and recruit the chromatin-remodeling protein HIRA to maintain the chromatin in a stable repressive state [15, 16, 39]. In mammalian cells, HIRA was shown to interact with a histone deacetylase [18]. Moreover, it was observed that Arabidopsis seedlings treated with TSA, an inhibitor of HDACs, produced abaxialized filamentous leaves, indicating the involvement of HDACs in leaf morphogenesis [24]. In this
Figure 6. AS1 and HDA6 bound to KNAT1, KNAT2, and KNATM promoters. (A) Diagrams of KNAT1, KNAT2, KNATM and the regions examined by ChIP. X and Y indicate regions examined by ChIP. Motif I and II are indicated by square and triangle, respectively. (B, C) Recruitment of AS1 to the KNAT1, KNAT2 and KNATM loci. Relative fold enrichment was calculated by dividing the amount of DNA immunoprecipitated from Col plants by that from the as1-1 or as2-1 plants and compared with input DNA. Plants were grown under LD conditions for 18 days. ACTIN2 and TUB2 were used as the negative controls. The values shown are means ± SD. (D) Recruitment of HDA6 to the KNAT1, KNAT2 and KNATM loci. Relative fold enrichment was calculated by dividing the amount of DNA immunoprecipitated with the anti-Myc antibody from Col plants by that from the HDA6-Myc transgenic plants and compared with input DNA. HDA6-Myc in Col is a transgenic line expressing a Myc-tagged HDA6 in Col background, whereas HDA6-Myc in as1-1 is a transgenic line expressing a Myc-tagged HDA6 in as1-1 mutants. (E) Recruitment of HDA6 to the KNAT1, KNAT2 and KNATM is independent of its catalytic activity. DNA fragments coimmunoprecipitated with the anti-Flag antibody relative to axe1-5 were analyzed by ChIP. HDA6 All 5 mut in axe1-5 is a transgenic line expressing HDA6 bearing the five amino acid mutation of the active site in axe1-5 mutants. Plants were grown under LD conditions for 18 days. ACTIN2 and TUB2 were used as the negative controls. The values shown are means ± SD.

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study, we provided direct evidence indicating that HDA6 is involved in leaf morphogenesis by interacting with AS1 and AS2 to regulate the KNOX expression. Compared with the single mutants, as1-1/axel-5 and as2-1/axel-5 double mutants show more severe phenotypes on curling leaves, petiole lengths, and leaflet-like structures, supporting that HDA6 acts with AS1 and AS2 to regulate leaf development. Taken together, our results demonstrated that histone deacetylation is one of the epigenetic components involved in AS1–AS2 complex-mediated KNOX repression. HDA6 may therefore be part of the AS1–AS2 repression complex to repress the target gene expression. Our data indicate that loss of one component of HDA6, AS1 and AS2 does not affect the interaction of two others in Arabidopsis. Previous studies indicated that the interaction between AS1 and AS2 is required for their binding to the promoters of KNOX genes, because neither AS1 nor AS2 alone was able to bind to the target DNA sequences in vitro [40]. We observed the loss of binding of AS1 to the KNOX chromatin in the as2-1 mutant, suggesting that AS2 is required for the AS1 binding. Furthermore, HDA6 cannot bind to KNOX chromatin in as1-1 mutants, indicating that AS1 is required to recruit HDA6. Taken together, both AS1 and AS2 are required for the recruitment of HDA6 to chromatin in repression of KNOX genes.

A recent work has also shown that the Polycomb Repressive Complexes (PRCs) repress KNOX transcription [40]. It was found that CLF-containing PRC2 regulates KNOX genes by trimethylation of histone H3K27 [41]. Thus, AS1 and AS2 may also recruit other chromatin factors such as PRCs to regulate class I KNOX genes. Taken together, our results suggested that HDA6 is one of the epigenetic components involved in the AS1–AS2 complex-mediated KNOX repression during leaf development in Arabidopsis.

Materials and Methods

Plant materials

Arabidopsis thaliana was grown in 23°C under LD (16 h light/8 h dark) or SD (8 h light/16 h dark) conditions. axel-5, sil1, as1-1 and as2-1 are in the Col background, whereas the HDA6 RNAi lines CS24038 and CS24039 are in Ws background.

Quantitative RT–PCR analysis

Arabidopsis leaves (0.2 g) were ground with liquid nitrogen in a mortar and pestle and mixed with 1 ml Triool Reagent (Invirogen) to isolate total RNA. After treated with DNase (Promega), two microgram of total RNA was used for the first-strand cDNA synthesis. cDNA was synthesized in a volume of 20 μl that contained the Moloney Murine Leukemia Virus Reverse Transcriptase buffer (Promega), 1.5 μM poly(dT) primer, 0.5 mM deoxyribonucleotide triphosphates, 25 units RNasin ribonuclease inhibitor, and 200 units Moloney Murine Leukemia Virus Reverse Transcraptase at 37°C for 1 h.

cDNAs obtained from reverse transcription were used as a template to run real-time PCR. The following components were added to a reaction tube: 9 μl of iQ SYBR Green Supermix solution (Bio-Rad), 1 μl of 5 μM specific primers, and 8 μl of the diluted cDNA template. Thermocycling conditions were 95°C for 3 minutes followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 20 s, with a melting curve detected at 95°C for 1 minute, 55°C for 1 minute, and detected the denature time from 55°C to 95°C. Each sample was quantified at least triplicate and normalized using Ubiquitin 16 as an internal control. The gene-specific primer pairs for quantitative RT-PCR are listed in Table S1.

ChIP assays

ChIP assay was carried out as described [42]. Chromatin extracts were prepared from 10 day old seedlings treated with formaldehyde. The chromatin was sheared to an average length of 500 bp by sonication and immunoprecipitated with specific antibodies including anti-acetylated histone H3K9K14 (Catalogue no. 06-599, Millipore), anti-trimethylated histone H3K4 (Catalogue no. 04-745, Millipore), anti-c-Myc (Catalogue no. M4439, Sigma) and anti-FLAG (Catalogue no. F1804, Sigma). The DNA cross-linked to immunoprecipitated proteins was analyzed by real-time PCR. Relative enrichments of various regions of KNAT1, KNAT2 and KNATM in axel-5, as1-1 and as1-1/axel-5 over Col were calculated after normalization to ACTIN2. Each of the immunoprecipitations was replicated three times, and each sample was quantified at least in triplicate. The primers used for real-time PCR analysis in ChIP assays are listed in Table S2.

BiFC assays

To generate the constructs for BiFC, full-length coding sequences of HDA6, AS1 and AS2 were PCR-amplified using Pfu polymerase (Finnzymes). The PCR products were subcloned into the pENTR/SD/D-TOPO or pCR3/GW/TOPO vector and then recombined into the pEarleyGate201-YN and pEarleyGate202-YC vectors [22]. The resulting constructs were transformed into the Agrobacterium GV3101 and the Agrobacteria containing these constructs were cotransfected into five week old Nicotiana benthamiana leaves. For the protoplast transient expression, HDA6, AS1 and AS2 were fused with pEarleyGate201-YN or pEarleyGate201-YC were co-transfected into protoplasts by PEG transfection [43]. Transfected leaves and protoplasts were imaged using TCS SP5 (Leica) Confocal Spectral Microscope Imaging System.

In vitro pull-down assays

Pull-down assays were performed as previously described [44] with some modifications. 2 μg Myelin basic protein (MBP) and MBP-AS1 recombinant proteins were incubated with 30 μl of MBP resin in a total volume of 500 μl of MBP binding buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA) for 2 h at 4°C, and the binding reaction was washed 3 times by the binding buffer, then 2 μg GST-HDA6 recombinant protein was added and incubated for additional 2 h at 4°C. After extensive washing (at least 8 times), the pulled down proteins were eluted by boiling, separated by 10% SDS-PAGE, and detected by western blotting using an anti-GST antibody.

Coimmunoprecipitation assays

Coimmunoprecipitation assays were performed as previous described [23]. The 20-day-old axel-5/35S:GFP-HDA6, axel-5 and as1-1 plants were harvested and ground in liquid nitrogen. Total proteins were extracted in an extraction buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl2, 1 mM DTT, 20% glycerol, and 1% CA-630) containing protease inhibitor cocktail (Roche). Cell debris was pelleted by centrifugation at 14,000 g for 30 min. The supernatant was incubated with anti-AS1 or anti-GFP specific antibody overnight at 4°C by gentle rotation, then 50 μl of protein G agarose beads (Millipore) was added. After 3 h of incubation at 4°C by gently rotation, the beads were centrifuged and washed five times with a washing buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl2, 1 mM DTT, 10% glycerol, and 1% CA-630). Proteins were eluted with 40 μl of 2× sample buffer and analyzed by western blotting using anti-AS1 and anti-GFP (Santa Cruz Biotechnologies) antibodies.
**Supporting Information**

**Figure S1** Negative controls of BiFC in N. benthamiana leaves. HDA6, AS1 and AS2 fused with VN or YC and the empty vector (YN and YC) were co-delivered into tobacco leaves as negative controls. No YFP signals were detected. The nucleus was stained with Hoechst nuclear stain (Blue).

**Figure S2** AS1 and AS2 formed the heterodimer in plants. BiFC in N. benthamiana leaves showing interaction between AS1 and AS2 in living cells. Arrows indicate nuclear fluorescence.

**Figure S3** Interaction among HDA6, AS1 and AS2 in the protoplasts of wild-type and mutants in Arabidopsis. (A) BiFC showing interaction between HDA6 and AS1 in wild type Col and as1-2/1 mutant plants. (B) BiFC showing interaction between HDA6 and AS2 in wild type Col and axl-5 mutant plants. (C) BiFC showing interaction between AS1 and AS2 in wild type Col and axl-5 mutant plants. (D) Negative controls of BiFC. HDA6, AS1 and AS2 fused with N-terminal (pEarleyGate201-YN) or C-terminal (pEarleyGate201-YC) were co-transformed into protoplasts of wild-type Col and mutants. VirD2NLS fused with mCherry was used as a nuclear marker (Blue).

**Figure S4** Leaf phenotype of HDA6-RNAi plants. WS and HDA6-RNAi (CS24038 and CS24039) plants were grown under SD conditions for 30 days. Both CS24038 and CS24039 plants displayed the margin serration and curling leaf phenotypes.

**Figure S5** The expression of AS1, AS2, PHB, PHV, CUC1, and CUC2 in axl-5 mutants. qRT-PCR analyses of gene expression in axl-5 plants grown under LD conditions for 20 days. The values shown are means ± SD.

**Table S1** Gene-specific primer pairs for quantitative RT–PCR.

**Table S2** Primers used for quantitative RT–PCR analyses in ChiP assays.

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**Author Contributions**

Conceived and designed the experiments: ML, C-WY, F-FC, KW. Performed the experiments: ML, C-WY, F-FC, LZ, GT. Analyzed the data: ML, C-WY, F-FC, XL, YC, J-YY, KW. Contributed reagents/materials/analysis tools: KW. Wrote the paper: ML, C-WY, KW.
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