Histone Deacetylases and ASYMMETRIC LEAVES2 Are Involved in the Establishment of Polarity in Leaves of Arabidopsis

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We show that two Arabidopsis thaliana genes for histone deacetylases (HDACs), HDT1/HD2A and HDT2/HD2B, are required to establish leaf polarity in the presence of mutant ASYMMETRIC LEAVES2 (AS2) or AS1. Treatment of as1 or as2 plants with inhibitors of HDACs resulted in abaxialized filamentous leaves and aberrant distribution of microRNA165 and/or microRNA166 (miR165/166) in leaves. Knockdown mutations of these two HDACs by RNA interference resulted in phenotypes like those observed in the as2 background. Nuclear localization of overproduced AS2 resulted in decreased levels of mature miR165/166 in leaves. This abnormality was abolished by HDAC inhibitors, suggesting that HDACs are required for AS2 action. A loss-of-function mutation in HASTY, encoding a positive regulator of miRNA levels, and a gain-of-function mutation in PHABULOSA, encoding a determinant of adaxialization, suppressed the generation of abaxialized filamentous leaves by inhibition of HDACs in the as1 or as2 background. AS2 and AS1 were colocalized in subnuclear bodies adjacent to the nucleolus where HDT1/HD2A and HDT2/HD2B were also found. Our results suggest that these HDACs and both AS2 and AS1 act independently to control levels and/or patterns of miR165/166 distribution and the development of adaxial-abaxial leaf polarity and that there may be interactions between HDACs and AS2 (AS1) in the generation of those miRNAs.

INTRODUCTION

The determination of cell fate along the adaxial-abaxial axis is essential for the development of leaves (Sussex, 1955; Waites and Hudson, 1995). Previous genetic analyses in Arabidopsis thaliana revealed that FILAMENTOUS FLOWER (FIL), YABBY3, KANADI1 (KAN1), KAN2, KAN3, ETTIN/AUXIN RESPONSE FACTOR3 (ARF3), and ARF4 play important roles in the determination of abaxial cell fate and the subsequent expansion of the lamina (Eshed et al., 1999, 2001, 2004; Sawà et al., 1999; Siegfried et al., 1999; Kerstetter et al., 2001; Pekker et al., 2005). By contrast, the extensive and enhanced accumulation in Arabidopsis leaf cells of the transcripts of the PHABULOSA (PHB), PHAVOLUTA (PHV), and REVOLUTA (REV) genes, in which target sites for microRNA165 and/or microRNA166 (miR165/166) had been disrupted, resulted in changes in cell fate from abaxial to adaxial (McConnell et al., 2001; Emery et al., 2003; Mallory et al., 2004). Therefore, these latter genes appear to be redundant positive regulators that are sufficient for the determination of adaxial cell fate, and the activity of these genes appears to be regulated by microRNA (miRNA). Kidner and Martienssen (2004) proposed that the adaxially restricted distribution of transcripts of PHB, PHV, and REV can be ascribed to that of miR165/166 on the abaxial side of leaf primordia. Surgical isolation of leaf primordia results in the outgrowth of abaxialized radial organs, suggesting that a meristem-derived signal directs adjacent regions of leaf primordia to adopt an adaxial fate (Sussex, 1955). Therefore, one of the most important cues for the establishment of adaxial-abaxial polarity in the development of leaves appears to be the regulation of the distribution of miR165/166. Thus, the existence has been proposed of a regulatory mechanism that involves miR165/166 on the adaxial side of leaves.

A loss-of-function mutation of the PHANTASTICA (PHAN) gene in Antirrhinum majus, in combination with low-temperature treatment or with the handlebars mutation, results in a defect in the adaxial development of leaves (Waites and Hudson, 1995, 2001). Arabidopsis plants with the recessive mutations asymmetric leaves1 (as1) and as2 exhibit various defects in leaf development (Rédei and Hirono, 1964; Serrano-Cartagena et al., 1999; Byrne et al., 2000; Semiarti et al., 2001; Xu et al., 2003). Moreover, the phenotype of as1 as2 double mutants is similar to that of each single mutant, suggesting that AS1 and AS2 function within the same pathway (Serrano-Cartagena et al., 1999). AS1 encodes a MYB protein, which is a homolog of the products of
the PHAN and ROUGH SHEATH2 (RS2) genes in maize (Zea mays; Byrne et al., 2000). AS2 encodes a protein that contains an AS2/LATERAL ORGAN BOUNDARY (LOB) domain (Iwakawa et al., 2002). In Arabidopsis plants with an erecta (er) background and an as1 or as2 mutation, leaf development is partially deficient on the adaxial side, suggesting that, similarly to PHAN, AS1 and AS2 are involved in the regulation of adaxial cell-fate determination (Xu et al., 2003). The involvement of AS2 in the adaxial-abaxial polarity has been also reported by Lin et al. (2003). The accumulation of miR165/166 is enhanced in as1 er and as2 er double mutants grown at high temperature (Li et al., 2005). However, we still do not know how AS1 and AS2 regulate the accumulation of miR165/166 and how they determine, in this way, fate of leaf cells.

The pickle (pk) mutation enhances the severity of the abnormal phenotype of as1 and as2 mutants (Ori et al., 2000). PKL encodes an ortholog of Mi-2, which belongs to the family of SWI2/SNF2 ATPase chromatin-remodeling factors (Eshed et al., 1999; Ogawa et al., 1999). Moreover, Mi-2 is also a component of the NuRD complex that is involved in transcriptional repression and includes two histone deacetylases (HDACs; Ahringer, 2000).

In this report, we show that the HDACs HDT1/HD2A and HDT2/HD2B are involved in the control of miRNA expression and the determination of cell fate in leaves of as2 mutant plants. We propose a model, based on our results, for the mechanism that underlies the establishment of polarity in Arabidopsis leaves.

RESULTS

Inhibition of HDAC Activity Results in Abaxialized Filamentous Leaves in as1 and as2 Mutant Plants

We treated as1 and as2 mutant plants with specific inhibitors of HDACs, namely, trichostatin A (TSA) and 4-[(dimethylamino)-N-[6-(hydroxyamino)-6-oxohexyl]-benzamide (CAY10398; CAY) at concentrations from 0 to 10 μM and 0 to 100 μM, respectively. On medium that contained 1 to 3 μM TSA or 10 to 30 μM CAY, the adaxial-abaxial polarity of leaves of wild-type plants was unaffected or only slightly affected. By contrast, as1 and as2 mutant plants frequently produced filamentous leaves with reduced numbers of trichomes (Figures 1A to 1F, Table 1) and trumpet-shaped leaves. This effect was dependent on the dose of inhibitor and the mutation (as1 or as2; Figures 1G and 1H) and was nonspecific to the allelic series examined (Table 1). Protein gel blot analysis revealed a stepwise increase in overall levels of acetylation of histone H4 in as2 plants after treatment with 1 to 3 μM TSA for 3 h (Figure 1I). These results were correlated with the severity of the mutant phenotype. Similar results were obtained with wild-type plants and as1 mutant plants (see Supplemental Figure 1 online). Medium containing 10 μM TSA or 100 μM CAY was lethal to wild-type, as1, and as2 plants. These results indicated that the efficacy of each drug was unaffected by the as1 and as2 mutations and that inhibition of HDAC activity enhanced the severity of the mutant phenotype of as1 and as2 mutant leaves specifically.

In the filamentous leaves, there was no obvious xylem system of the type that is normally localized adaxially in leaf vascular bundles (cf. Figure 1D to 1F). Strong signals from green fluorescent protein (GFP), encoded by a gene for GFP under the control of the FIL promoter (Watanabe and Okada, 2003) and designated FILp:GFP, were detected in cells at peripheral positions in such filamentous leaves (Figures 1K and 1L), whereas no signals were detected in cells on the adaxial side of leaves of wild-type plants or of as2 mutant plants in the absence of these inhibitors (Watanabe and Okada, 2003; Figure 1J). We obtained similar results with the as1 mutant (Figure 1M). Furthermore, scanning electron microscopy revealed that the epidermal cells of filamentous leaves on as2-1 mutant plants grown in the presence of TSA were long and narrow (Figure 1N), resembling those of transgenic plants in which KAN2 is ectopically expressed (Eshed et al., 2001) and of as2 er double mutant plants grown at high temperature (Xu et al., 2003). These results suggested that the filamentous leaves that formed on as1 and as2 mutant plants in the presence of HDAC inhibitors had been abaxialized (Figure 1O).

The Distribution of miR165/166 Is Aberrant in Leaves of as1 and as2 Mutant Plants Grown on Medium with TSA

To monitor the accumulation and activity of miR165/166 in situ, we constructed a reporter gene (Figure 2). This reporter gene included erG3 GFP (Tamai and Meshi, 2001) for targeting to the endoplasmic reticulum and a sequence complementary to miR165 (c165) in the 3‘ untranslated region. The gene was fused to the 3SS promoter of Cauliflower mosaic virus (3SS; Figure 2A). We also constructed two control reporter genes. One of them had a point mutation that mimics that of phb-3D (McConnell et al., 2001) in the region of c165 and the other did not include c165. The constructs were designated 3SS:erG3 GFP:c165, 3SS:erG3 GFP:mc165, and 3SS:erG3 GFP, and each was introduced into wild-type plants (Columbia-0 [Col-0]).

In sections of a 3SS:erG3 GFP plant (line #3.2), the signal due to GFP was detected constitutively (Figure 2D). At a less-sensitive level of detection (Figures 2B and 2C), the signal was relatively strong in vascular bundles and the epidermis, and no difference between the adaxial and abaxial sides of the leaf was evident. Similar results were obtained for 3SS:erG3 GFP:mc165. By contrast, the signal on the adaxial side was stronger than on the abaxial side of the leaf in a 3SS:erG3 GFP:c165 plant (line #2.1) at three different levels of sensitivity (Figures 2E to 2G), suggesting the prominent accumulation of miR165/166 on the abaxial side of wild-type leaves. Similar patterns of fluorescence were observed in another Col-0 transgenic line (line #2.2) of 3SS:erG3 GFP: c165 plants and independent lines of as2-1 3SS:erG3 GFP:c165 plants (described below), suggesting that these patterns of GFP fluorescence were not specific to the first-mentioned transgenic line.

Signals due to GFP in cells of young leaves of as2-1 3SS:erG3 GFP:c165 plants (line #1.1), grown on medium without inhibitors, were also much stronger on the adaxial than on the abaxial side (Figure 2H), resembling those of 3SS:erG3 GFP: c165 plants (Figures 2E to 2G). By contrast, signals from the filamentous leaves of as2-1 3SS:erG3 GFP:c165 plants, grown on medium with TSA, were weaker on the adaxial side and limited to central regions (Figure 2I). Similar results were obtained with
Dose-dependent accumulation of hyperacetylated histone H4 in were grown for 17 d on medium that contained TSA or CAY as indicated on horizontal axes. The filamentous leaves on each plant were counted.

was performed with antibodies specific for acetylated histone H4 (AcH4) and histone H4 (H4).

and  by dexamethazone (Dex) and by mifepristone (RU486; RU). A

To investigate the function of AS2 in the nuclei (Iwakawa et al., 2002), we introduced, into Col-0 plants, a chimeric gene that encoded the ligand binding domain of the glucocorticoid receptor (GR) fused to the C terminus of AS2, in frame, under the control of the 35S promoter. We designated this gene 35S:AS2-GR (Figure 3A). The nuclear localization of AS2-GR was induced by dexamethazone (Dex) and by mifepristone (RU486; RU). A

another line of as2-1 35S:erG3GFP:c165 plants (line #1.2) and with independent transgenic as2-1 35S:GUS:c165 lines (lines #G1.1 and #G3.1; see Supplemental Figures 2A to 2D online), suggesting that the ectopic localization of miR165/166 occurred on the adaxial side of young leaves as a result of the combination of the as2 mutation and inhibition of HDACs.

AS2 Protein Functions in Nuclei

To investigate the function of AS2 in the nuclei (Iwakawa et al., 2002), we introduced, into Col-0 plants, a chimeric gene that encoded the ligand binding domain of the glucocorticoid receptor (GR) fused to the C terminus of AS2, in frame, under the control of the 35S promoter. We designated this gene 35S:AS2-GR (Figure 3A). The nuclear localization of AS2-GR was induced by dexamethazone (Dex) and by mifepristone (RU486; RU). A chimeric protein, in which GFP and GR were fused to the C terminus of AS2, designated AS2-GFP-GR, was concentrated in the nucleus after treatment with Dex of tobacco (Nicotiana tabacum) BY-2 cultured cells that had been transformed with 35S:AS2-GFP-GR (see Supplemental Figure 3 online). We generated 35S:AS2-GR transgenic Arabidopsis plants (line #5.4) and introduced the 35S:AS2-GR construct into as2-1 plants by genetic cross. The resulting plant was designated as2-1 35S:AS2-GR (line #5.4). After treatment with Dex, as2-1 35S:AS2-GR plants had normal leaves (see Supplemental Figure 4 online). When the conserved Gly residue (Iwakawa et al., 2002) in the AS2-GR encoded by the transgene was replaced by Glu, restoration of normal leaf shape did not occur (see Supplemental Figure 4 online). Therefore, we concluded that the AS2-GR fusion protein was functional in the nuclei of as2-1 35S:AS2-GR plants.
Table 1. Frequencies of Radialized Leaves on Plants after Growth on Medium That Contained an Inhibitor of HDACs

<table>
<thead>
<tr>
<th>Genotype</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>&gt;3</th>
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<td>Col-0</td>
<td>105 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>105</td>
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<tr>
<td>as1-1</td>
<td>118 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>118</td>
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<tr>
<td>as2-1</td>
<td>128 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<tr>
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<tr>
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<td>124 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<tr>
<td>Col-0</td>
<td>85 (93%)</td>
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<td>2 (2%)</td>
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<td>91</td>
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<td>as1-1</td>
<td>27 (22%)</td>
<td>34 (28%)</td>
<td>47 (39%)</td>
<td>7 (6%)</td>
<td>6 (5%)</td>
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<td>11 (10%)</td>
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<td>as2-4</td>
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<td>58 (56%)</td>
<td>10 (10%)</td>
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<tr>
<td>Col-0</td>
<td>87 (91%)</td>
<td>7 (7%)</td>
<td>2 (2%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>96</td>
</tr>
<tr>
<td>as1-1</td>
<td>22 (21%)</td>
<td>27 (25%)</td>
<td>28 (26%)</td>
<td>26 (25%)</td>
<td>3 (3%)</td>
<td>106</td>
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<td>20 (20%)</td>
<td>27 (27%)</td>
<td>13 (13%)</td>
<td>23 (23%)</td>
<td>99</td>
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<tr>
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<td>1 (3%)</td>
<td>1 (3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>36</td>
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</table>

The filamentous or trumpet-shaped leaves on each plant were counted under a stereomicroscope 17 to 21 d after imbibition of seeds. Ler, Landsberg erecta.

Nuclear Localization of AS2-GR Protein Represses the Accumulation of miR165/166

Ectopic expression of AS2 results in upwardly curling leaves (Iwakawa et al., 2002; Lin et al., 2003). The phenotype resembles that of plants with mutations in genes for either the biogenesis of miRNAs or the target sites for miR165/166. To examine the effect of nuclear localization of AS2-GR protein on the distribution of miR165/166, we generated 35S:AS2-GR (from line #5.4) 35S:erG3GFP:c165 (from line #2.1) double-transgenic plants by genetic cross. In leaves of the 35S:AS2-GR plants that had been treated with RU, strong signals due to expression of GFP extended to the abaxial side of leaves (Figures 3B and 3C), suggesting that levels of miR165/166 had been reduced. Transcripts of PHB were confined to the adaxial side of young leaf primordia in wild-type plants (Figure 3D). They were also found on the abaxial side of leaf primordia in 35S:AS2-GR plants treated with RU (line #5.4; Figure 3E). These results suggested that the nuclear localization of AS2-GR has suppressed expression of miR165/166 even on the abaxial side of leaves, with resultant ectopic accumulation of PHB transcripts.

Upward Curling of Leaves and the Reduction in Levels of miR165/166 Observed in 35S:AS2-GR Transgenic Plants Depend on Endogenous AS1 and HDAC Activity

In the presence of 10 nM Dex or 1 μM RU, 35S:AS2-GR transgenic plants (line #5.4) had severely upwardly curled leaves (Figure 4A), while 100 nM Dex was lethal. Similar results were obtained from four other independent transgenic lines (lines #1, 2, 3.4, and 4). To examine whether the phenotype depended on AS1, we introduced 35S:AS2-GR (from line #5.4) into as1-1 mutants by genetic cross. In the presence of 10 nM Dex, leaves of as1-1 35S:AS2-GR plants did not curl upwards (Figure 4B), unlike those of 35S:AS2-GR plants (Figure 4A), but 100 nM Dex was still lethal. TSA also reduced the number of upwardly curling leaves in 35S:AS2-GR plants (line #5.4; Figure 4C). These results suggested that upwardly curling leaves required both endogenous AS1 and HDAC activity.

Figure 2. The 35S:erG3GFP:c165 Reporter Gene, Which Revealed the Distribution of miR165/166, Demonstrated the Aberrant Accumulation of miR165/166 in as2 Mutant Plants That Had Been Treated with TSA. (A) Schematic representation of the 35S:erG3GFP:c165 and 35S:erG3GFP:mc165 constructs. Sequences of c165, mc165, miR165, and miR166 are shown. In the mc165 sequence, the adenine (A) that replaced guanine (G) in the c165 sequence is underlined. (B) to (G) Fluorescent signals due to 35S:erG3GFP that were restricted to the central region of a young leaf of an as2-1 transgenic plant with a Col-0 background. Three pictures were taken of individual sections from each panel at different sensitivities. The sensitivity for detection of fluorescence due to GFP was increased gradually from the top to the bottom photograph in each column to allow visualization of overall patterns of fluorescence. Bars = 50 μm. (H) and (I) as2-1 35S:erG3GFP:c165 (line #1.1) grown on medium without or with (I) 3 μM TSA. Signals due to GFP that were restricted to the central region of a young leaf of an as2-1 35S:erG3GFP:c165 plant treated with TSA are indicated by an arrow. Green, fluorescence due to GFP; red, autofluorescence. Bars = 50 μm.
As suggested by the results obtained with the 35S:erG3GFP:c165 reporter gene in the 35S:AS2-GR plants (Figure 3C), levels of endogenous miR165/166 were indeed depressed in the 35S:AS2-GR plants after treatment with RU or Dex (Figure 4D). The as1 and TSA each restored normal levels of miR165/166 in the 35S:AS2-GR plants (line #5.4; Figure 4D). We obtained similar results with another transgenic line (line #3.4). Thus, AS2-GR in the nucleus requires AS1 for a reduction in levels of mature miR165/166, as does endogenous AS2. Accumulation of the precursors to miR165/166 (pri-miR165/166) in 35S:AS2-GR plants was unaffected by treatment with RU (Figure 4E). We also examined levels of miR171, miR172, and miR173 in 35S:AS2-GR plants grown in the presence of RU. The accumulation of miR171 and miR173 was unaffected in 35S:AS2-GR plants, but that of miR172 was reduced (see Supplemental Figure 5 online).

We determined how quickly changes in miR165/166 occur following Dex treatment of 35S:AS2-GR plants. Incubation of these plants in a liquid culture containing 10 μM Dex for 24 h did not affect levels of miR165/166. Subsequently, we examined whether longer incubations of 35S:AS2-GR plants on a solid medium may affect levels of miR165/166. Results of this experiment showed that the levels decreased 4 d after the incubation (see Supplemental Figure 6 online), suggesting that the effect on decrease of miR165/166 by the nuclear localization of AS2-GR is likely to be indirect.

Identification of HDT1/HD2A and HDT2/HD2B as the Relevant HDACs

The Arabidopsis genome includes 12, four, and two genes for members of the RPD3/HDA1, HD2, and SIR2 families, respectively, of HDACs (Pandey et al., 2002). Proteins belonging to the SIR2 family are insensitive to TSA, while members of the RPD3/ HDA1 and HD2 families are very susceptible to TSA and CAY (Jung et al., 1997). To identify the HDAC(s) responsible for the determination of the leaf polarity, we constructed RNA interference (RNAi) libraries specific for the genes in the RPD3/HDA1 and HD2 families, designating our libraries the HDA-RNAi library and the HDT-RNAi library, respectively. Then, we transformed as2-1 35S:AS2-GR (from line #5.4) plants, generated by genetic cross, with each library as described in Methods.

We looked for knockdown transformants with radialized (filamentous or trumpet-shaped) leaves (Figure 5A). Among 20 independent transformants obtained with the HDT-RNAi library, two knockdown lines (lines #1 and #301) had produced clearly radialized leaves in the T1 generation (as shown in the example in Figure 5B). A total of 104 of 129 plants (81%) and 42 of 83 plants (51%) of knockdown lines #1 and #301, respectively, produced radialized leaves in the T2 generation (Table 2). Analysis by PCR revealed that RNAi constructs specific for HDT1/HD2A and HDT2/HD2B and for HDT2/HD2B were present in the genomes of lines #1 and #301, respectively (Table 2). DNA analysis of other lines that formed radialized leaves in the T2 generation revealed that these lines contained T-DNA for RNAi of HDT1/HD2A (HDT1i) or T-DNA for RNAi of HDT2/ HD2B (HDT2i) (Table 2). Moreover, endogenous levels of HDT1/ HD2A and HDT2/HD2B transcripts in knockdown line #1 were lower than those in control host plants and in siblings in the T2 generation without radialized leaves, whereas those of HDT3/ HD2C and HDT4/HD2D were not depressed (Figure 5C). We backcrossed knockdown line #1 to a Col-0 plant to examine the phenotype on a wild-type AS2 background. All F2 siblings, which were indistinguishable from wild-type plants, lacked radialized leaves. Therefore, we concluded that the phenotype was dependent on as2. However, both HDT1i and HDT2i were tightly linked to the radialized-leaf phenotype (see Supplemental Table 1 online). These results indicated that the formation of radialized leaves depended on HDT1i, HDT2i, or both in line #1.

To confirm our results, we reintroduced HDT1i and HDT2i into as2-1 35S:AS2-GR plants. Among seven lines of newly isolated transformants, one transformant (T1 generation), which was sterile, had filamentous leaves similar to those of line #1. From 9 to 23% of T2 siblings with radialized leaves were segregated from another three lines among the seven lines. These results suggested that HDT1/HD2A and/or HDT2/HD2B might be the relevant factor(s). Moreover, they were consistent with our earlier observation that the generation of filamentous leaves depended on both the as2 or as1 mutation and the dose of HDAC inhibitor (Figure 1, Table 1).
Among 67 independent transgenic lines obtained from the HDA-RNAi library, we found no knockdown transformants with radIALIZED leaves in the T1 and T2 generations.

HDT1/HD2A and HDT2/HD2B Are Involved in the Determination of Adaxial-Abaxial Polarity

We examined the expression of genes involved in the determination of adaxial-abaxial polarity by RT-PCR in knockdown line #1 (as2-1 35S:AS2-GR [from line #5.4]; hdt1-RNAi hdt2-RNAi). In T2 siblings that formed filamentous leaves, levels of transcripts of PHB and FIL were lower and higher, respectively, than those in the normal (nonfilamentous) siblings (Figure 5C). Taken together, these observations suggested that the levels of transcripts of the PHB and FIL genes were affected positively and negatively, respectively, by HDT1/HD2A and HDT2/HD2B in leaves with the as2 mutant background.

On the surfaces of filamentous leaves on plants of knockdown lines #1 and #301, the epidermal cells were long and narrow, with reduced numbers of trichomes (Figures 5D and 5E). These surface characteristics of filamentous leaves were strikingly similar to those of as2-1 plants that had been exposed to TSA (Figure 1N).

Transcripts of the FIL gene accumulate throughout younger leaf primordia, and their levels on the adaxial side diminish as primordia grow (Sawa et al., 1999; Siegfried et al., 1999). The decrease in the accumulation of FIL transcripts that we observed in Col-0 (Figure 5F) was not evident in knockdown line #301 at the same stage (Figure 5G). A higher number of strong signals were observed preferentially on the adaxial side of the leaf primordium. Similar results were obtained from knockdown line #1. These results indicated that the filamentous leaves that were formed on plants of knockdown lines #1 and #301 had been abaxialized. Therefore, we concluded that HDT2/HD2B (and also HDT1/HD2A) were involved in the establishment of adaxial fate on the as2 mutant background.

Genetic Interaction between AS2 and Genes Related to the Function of miR165/166

We examined the effects of TSA on as2-1 hst-1, as2-1 phb-1D, and as1-1 hst-1 double mutants. In hst mutants, the levels of
Many miRNAs, including those of miR165/166, were lower than those in wild-type plants (Park et al., 2005). On medium without TSA, as2-1 hst-1 double mutants had undulating but nearly flat leaves, while as2-1 single mutants had downwardly curling leaves, and hst-1 mutants had upwardly curling leaves (Figures 6A to 6C). Even on medium with TSA, on which most as2-1 single-mutant plants formed abaxialized filamentous leaves, as2-1 hst-1 double mutants had flat leaves, as did hst-1 single mutants (Figures 6D to 6F). These observations showed that hst suppressed, at least to some extent, the effects of the as2 mutation and the inhibition of HDAC activity. The as1-1 hst-1 double mutant plants had a similar phenotype. Our results suggested that the formation of filamentous leaves by mutation of AS1 or AS2 and by TSA requires the activity of HST.

Regulation by miR165/166 of the expression of phb-1D transcripts is eliminated by disruption of the complementary sequences in the transcripts (McConnell et al., 2001; Mallory et al., 2004). Our as2-1 phb-1D double mutants formed filamentous leaves with many trichomes, and these leaves were indistinguishable from leaves of phb-1D single mutants, even in the presence of TSA (Figures 6G and 6H). On the surfaces of filamentous leaves on as2-1 phb-1D plants, the shapes and organization of epidermal cells were somewhat irregular (Figure 6I), resembling those on the adaxialized leaves of phb-1D mutants (McConnell and Barton, 1998), as compared with those on abaxialized leaves on as2-1 plants in the presence of TSA and on HDT2/HDT2B knockdown lines with the as2-1 background (Figures 1N, 5D, and 5E). Thus, the phb-1D allele was epistatic to the as2-1 allele regardless of the presence of TSA.

**Subnuclear Localization of HDT1/HD2A, AS2, and AS1**

To examine the subnuclear localization of AS2 and AS1, we prepared plasmids that encoded the following constructs: yellow fluorescent protein (YFP) fused to the C terminus of AS1 (YFP-AS1) under the control of the 35S promoter frame, (GFP-AS1) under the control of an estrogen-inducible fluorescent protein (YFP) fused to the C terminus of AS2, in combination with the HPT gene and of a gene for hygromycin phosphotransferase (HPT), as a marker for the presence of T-DNA, were examined. F, filamentous; N, not filamentous.

<table>
<thead>
<tr>
<th>Line No.</th>
<th>RadIALIZED LEAVES on T1 Plants</th>
<th>T-DNA for RNAi</th>
<th>Frequency of Plants with RadIALIZED LEAVES among the T2 Progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Filamentous and trumpet-shaped</td>
<td>HDT1 and HDT2</td>
<td>81% (n = 129)</td>
</tr>
<tr>
<td>2</td>
<td>Unclear</td>
<td>HDT1</td>
<td>0% (n = 55)</td>
</tr>
<tr>
<td>3</td>
<td>Unclear</td>
<td>HDT2, HDT3, and HDT4</td>
<td>5% (n = 59)</td>
</tr>
<tr>
<td>4</td>
<td>Unclear</td>
<td>HDT1</td>
<td>0% (n = 30)</td>
</tr>
<tr>
<td>5</td>
<td>Unclear</td>
<td>HDT4</td>
<td>0% (n = 30)</td>
</tr>
<tr>
<td>6</td>
<td>Unclear</td>
<td>HDT4</td>
<td>0% (n = 30)</td>
</tr>
<tr>
<td>7</td>
<td>Unclear</td>
<td>HDT3</td>
<td>0% (n = 37)</td>
</tr>
<tr>
<td>8</td>
<td>Unclear</td>
<td>HDT3</td>
<td>0% (n = 30)</td>
</tr>
<tr>
<td>9</td>
<td>Unclear</td>
<td>HDT3</td>
<td>0% (n = 30)</td>
</tr>
<tr>
<td>10</td>
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<td>HDT3</td>
<td>0% (n = 30)</td>
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<td>12</td>
<td>Unclear</td>
<td>HDT1 and HDT4</td>
<td>9% (n = 34)</td>
</tr>
<tr>
<td>301</td>
<td>Trumpet-shaped</td>
<td>HDT2</td>
<td>51% (n = 83)</td>
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</table>

Both the analysis of the DNA of T1 plants and the segregation analysis of T2 siblings were performed with 11 of 20 transgenic lines. No obvious abnormalities, apart from filamentous or trumpet-shaped leaves, were observed in the T1 and T2 generations of the various knockdown lines.
(YFP-HDT1) under the control of the 35S promoter (35S:YFP-HDT1); and GFP fused to the C terminus of nucleolin, in frame, (Nuc.-GFP) under the control of the 35S promoter (35S:Nucleolin-GFP), as a marker of nucleoli.

Fluorescent signals due to AS2-YFP protein were localized as subnuclear bodies around the periphery of nucleoli in the leaf epidermal cells of ER:AS2-YFP (from line #T1.1) 35S:Nucleolin-GFP (from line #T9) double-transgenic plants (98% [n = 47]; see examples in Figures 7B and 7C). Similar results were obtained from another transgenic line ER:AS2-YFP (#T10.5) plants. Fluorescent signals due to GFP-AS1 protein were localized as one to several subnuclear bodies in the leaf epidermal cells of 35S:GFP-AS1 (lines #T11 and #T12) transgenic plants (86% [n = 29]). The largest bodies were localized around the periphery of nucleoli (see Supplemental Figure 7 online). As reported previously by Lawrence et al. (2004), our results also showed that fluorescent signals due to YFP-HDT1 were localized in the nucleoli in 35S:YFP-HDT1 (lines #T13 and #T15) transgenic plants (see Supplemental Figure 7 online). When cells that were positive for both GFP and YFP signals in the ER:AS2-YFP (from line #T11.1) 35S:GFP-AS1 (from lines #T17) double-transgenic plants were examined, fluorescent signals from GFP-AS1 and AS2-YFP were colocalized around the periphery of nucleoli (100%; Figures 7D to 7F). The proportion of such double-positive cells was 20% of AS2-YFP positive cells and variable in GFP-AS1 positive cells depending on conditions for the induction of AS2-YFP gene expression. Using another double-transgenic line, ER:AS2-YFP (from line #T11.1) 35S:GFP-AS1 (from line #T12), similar results were obtained.

The subnuclear accumulation of AS1/AS2 appeared to be separate from the nucleolus, in which HDT1/HD2A and HDT2/HD2B were concentrated (Figure 7F; Lawrence et al., 2004; Zhou et al., 2004).

**DISCUSSION**

HDACs and AS2 (AS1) Are Involved Independently in the Establishment of the Adaxial-Abaxial Polarity of Leaves in Arabidopsis

In this study, we found that loss of function of two genes for HDAC, HDT1/HD2A and HDT2/HD2B, on the as1 or as2 mutant background resulted in abaxialized filamentous leaves and aberrant distribution in leaves of miR165/166 (Figures 1, 2, and 5). These observations suggest that these HDACs are involved in the determination of adaxial-abaxial polarity in leaves, as are AS1 and AS2 (Xu et al., 2003). Inducible nuclear localization of overproduced AS2-GR in Arabidopsis resulted in upwardly curled leaves, a reduction in levels of miR165/166, and the aberrant accumulation of transcripts of PHB in the abaxial domain (Figures 3 and 4). These abnormalities were suppressed by inhibition of HDACs and the as1 mutation. Thus, the phenotype generated by AS2 seems to require the functions of the HDACs and AS1. It was demonstrated recently that AS2 transcripts accumulate in the

![Figure 6](image-url)
Upward Curling of Leaves Seems to Be Closely Related to the Biogenesis and/or Functions of Some miRNAs

Upward curling is one of the most obvious phenotypic abnormalities associated with the nuclear import of overproduced AS2 (Figure 4; Iwakawa et al., 2002; unpublished data). Since loss-of-function mutations in the AS2 genes result in downward curling of leaves (Semiarti et al., 2001; Iwakawa et al., 2002; unpublished data) and since overexpression of some other members of the AS2/LOB family does not induce such conspicuous upward curling while most members of this family have no effect at all on the curling status of leaves (Shuai et al., 2002; Nakazawa et al., 2003; Chalfun-Junior et al., 2005), the upward curling observed upon overproduction and nuclear localization of AS2 seems to be attributable to an intrinsic function of AS2.

Several genes are known that are related to the formation of upwardly curled leaves. Loss-of-function mutations in the HST, HUA ENHANCER1 (HEN1), DCL1/_CARPEL FACTORY, HYPOASTIC LEAVES1 (HYL1), SERRATE, and ARGONAUTE1 (AGO1) genes, all of which are related to the formation or functions of miRNA, result in the upward curling of leaves (Telfer and Poethig, 1998; Jacobsen et al., 1999; Lu and Fedoroff, 2000; Chen et al., 2002; Han et al., 2004; Vaucheret et al., 2004; Grigg et al., 2005; Mallory and Vaucheret, 2006). The mutations reduce levels of miRNAs and increase levels of transcripts of the PHB, PHV, and REV genes, which are targets of miR165/166 (Reinhart et al., 2002; Vaucheret et al., 2004; Vazquez et al., 2004; Grigg et al., 2005; Park et al., 2005; Yang et al., 2006). A point mutation, located at the site of cleavage mediated by miR165/166 within the INCURVATA4/ CORONA/ATHB-15 gene, also resulted in upcurled leaves (Ochando et al., 2006). These observations suggest that products of genes that are involved in the production and functioning of miR165/166 regulate leaf flatness, apparently opposing the functions of AS2 and AS1.

The Mechanism whereby HDACs and AS2 (AS1) Control Adaxial-Abaxial Polarity

HDACs Are Involved in Establishing the Adaxial Domain by Affecting the Level and Patterns of Distribution of miR165/166 during Leaf Development in as2 Plants

Three types of model might explain how HDACs establish the adaxialization of leaves and control the distribution of miR165/166 in the as2 mutant. (1) The HDACs could affect the distribution of miR165/166, which could in turn establish adaxialization. (2) The HDACs could directly control leaf polarity, which could in turn alter the distribution of miR165/166. (3) The HDACs could control both processes separately. The second and third models seem less plausible than the first since the formation of abaxialized filamentous leaves, which was induced by inhibitors of HDACs and the as2 mutation, was suppressed by the hst-1 mutation (Figure 6). In addition, when double mutant (as2-1 phb-1D) plants, with a dominant allele associated with resistance to degradation of mRNA by miR165/166, were treated with inhibitors of HDACs, adaxialized filamentous leaves were formed instead of abaxialized leaves (Figure 6). Thus, the hst-1 and phb-1D mutations were epistatic to the inhibition of HDACs in the as2 mutant. It seems likely that the HDACs are involved in establishing the adaxial domain via effects on the levels and distribution of miR165/166 during leaf development in the as2 mutant. However, it remains unknown whether such effects in as2 mutants are directly or indirectly attributable to HDACs.

Since overexpression of AS2, as2 mutations, and application of inhibitors of HDACs had no obvious effects on levels of transcripts of AGO1, DCL1, HEN1, HST, and HYL1, which are required for biogenesis of miRNAs (our unpublished data), it seems to be unlikely that HDACs and AS2 are involved in the transcription of these genes or in the processes that are mediated by their products.

Possible Control by HDACs and AS2 (AS1) of Levels of miRNA165/166

Our results indicate that two genes for HDACs, HDT1/HDT2A and HDT2/HDT2B, function in establishment of the adaxial domain of leaves in Arabidopsis with the as2 mutation. The AS2 gene also plays a similar role in leaf development when the activity of the HDACs is reduced by an inhibitor or by knockdown of genes for...
HDACs by RNAi. As discussed above, it is likely that HDACs and AS2 are involved independently in the accumulation and/or distribution of miR165/166 in leaves of wild-type Arabidopsis. A similar function for AS2 was proposed by Li et al. (2005). Since nuclear localization of overproduced AS2 proteins did not affect levels of pri-miR165 or pri-miR166 but reduced levels of mature miR165/166 (Figure 4), it seems possible that AS2 might be responsible for posttranscriptional processing that generates mature miR165/166.

It is generally accepted that HDACs are involved in the control of transcription via the deacetylation of histones in chromatin. HDT1/HDA2A is involved in the deacetylation of nucleosomal histone H3 and in modulating transcription of genes for rRNA in genetic hybrids (Lawrence et al., 2004). In addition, HDT1/HDA2A and HDT2/HDA2B have been shown to repress the transcription of reporter genes (Wu et al., 2000, 2003). We found a relationship between the formation of abaxialized filamentous leaves and an increase in the level of acetylation of histone H4, both of which were induced by the application of TSA, an inhibitor of HDACs (Figure 1). In addition, application of TSA to the as2 mutant resulted in a slight increase in levels and a change in the distribution of mature miR165/166 (Figure 2). It seems plausible that HDACs might influence levels and patterns of distribution of these miRNAs by controlling the histone-deacetylation status of the chromatin.

The subnuclear localization of HDT1/HDA2A, HDT2/HDA2B, AS2, and AS1 allows us to speculate about possible functional relationships among these proteins. GFP-tagged AS1 and YFP-tagged AS2 were colocalized in subnuclear bodies adjacent to the nucleolus of epidermal cells of Arabidopsis leaves (Figure 7). The GFP-fused RS2 protein, a maize homolog of AS1, exhibits a similar pattern of subnuclear localization in Arabidopsis root cells (Theodoris et al., 2003). HDT1/HDA2A and HDT2/HDA2B are concentrated in the nucleoli of epidermal cells in Arabidopsis leaves (Zhou et al., 2004). Recently, factors such as RNA polymerase I/β and ARGONAUT4 that are required for gene silencing-related chromatin modification guided by small interfering RNAs (siRNAs) have been reported to colocalize with siRNAs within specific bodies in the nucleolus, which are proposed to function as a center for the assembly of complexes, facilitating siRNA-directed gene silencing at target loci (Semiarti et al., 2001). These HDACs and AS2 (AS1) were concentrated in the nucleolus and juxtaposed subnuclear bodies, respectively. The repression of miR165/166 accumulation by AS2-GR required the incubation of the 3SS:AS2-GR plants for 4 d (see Supplemental Figure 6 online), suggesting that the repression was probably indirect. The repression might occur at the posttranscriptional level (Figure 4E). Other unknown mechanisms (e.g., siRNA-directed gene silencing) might also mediate the repression of miRNA accumulation by AS2 (AS1) and HDACs. We need now to investigate the roles of the nucleolus and the subnuclear bodies in the mechanism that controls the adaxial-abaxial polarity.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana hst-1 and phb-1D mutants were obtained from the ABRC. Other plant materials used in this study are listed in Supplemental Table 2 online. Plants were grown on Murashige and Skoog (MS) medium with and without TSA (Wako), CAY10398 (Cayman Chemical), Dex (Sigma-Aldrich), or RU486 (Sigma-Aldrich) under previously described conditions (Semiarti et al., 2001) except during screening after introduction of the HDA-RNAi or HDT-RNAi library.

Histological Analyses

This sections and cleared specimens were prepared as described previously (Semiarti et al., 2001). For analysis of fluorescence due to GFP, hand-cut sections were examined by confocal microscopy as described previously (Watanabe and Okada, 2003). For staining of β-glucuronidase (GUS) activity, tissues were incubated at 30°C for ~16 h with staining solution (2 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 10% [v/v] dimethylformamide, 50 mM sodium phosphate buffer, pH 7.0, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 0.2% [w/v] Triton X-100, and 1 mM EDTA). Samples were dehydrated through an ethanol series and embedded in Technovit 7100 resin (Heraeus Kulzer) and sectioned at a thickness of 4 μm with a microtome. Scanning electron microscopy was performed as described previously (Semiarti et al., 2001).

Detection of Acetylation of Histone H4 by Protein Gel Blot Analysis

Seeds of as2-1, as1-1, and Col-0 plants were allowed to germinate on solid MS medium for 24 h in the light and then for 72 h in darkness. Eluted seedlings were dipped in liquid MS medium that contained 2% (w/v) sucrose and 0 to 3 μM TSA for 3 h. The histone-enriched fraction was prepared as described by Moehs et al. (1988). Antibodies against acetylated histone H4 (#06-866) and against histone H4 (#07-108) were purchased from Upstate.

RNA Gel Blot Analysis of miRNA

Total RNA was prepared as described elsewhere (Kim et al., 2005), and other procedures were performed according to the instruction manual provided by Ambion with the UltraHybOligo hybridization buffer and a locked nucleic acid (LNA)–containing oligonucleotide probe (Valöczy et al., 2004; Thermo Fisher Scientific), which had been labeled with T4 polynucleotide kinase and [γ-32P]ATP. The sequences of LNA-containing probes were as follows: pU695 for miR165/166, 5′-GGggGGAgGTAAG-CCTgGTCcGA-3′; pU706 for miR171, 5′-GAATTTgGCgGCGcCAAACA-3′; pU743 for miR172, 5′-ATgCAGAcTCAaaGATICT-3′; and pU744 for miR173, 5′-GAtATTcTCTCgGAcGAA-3′. Upper- and lowercase letters indicate DNA and LNA, respectively.

RNA Hybridization in Situ

RNA hybridization in situ was performed as described previously (Kaya et al., 2001) with some modifications. The plasmids for the preparation of the PHB and FIL probes were generous gifts from Hirokazu Tanaka (Universität Tübingen) and Shinichiro Sawa (University of Tokyo), respectively. Antisense probes for PHB and FIL were synthesized using T3 and T7 RNA polymerase, respectively, and digoxigenin-11-UTP (DIG-11-UTP). After hybridization at 50°C, sections were washed twice for 1 h at 55°C in 0.2× SSC. After the second wash, sections were treated with 20 μg/mL RNaseA in NTE (0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) for 30 min at 37°C and washed twice again for 5 min each in NTE at 37°C. Alkaline phosphatase–conjugated antibodies against DIG (Roche) and WesternBlue (Promega) were used for immunological detection of DIG.

Construction of RNAi Libraries

HDA10 and HDA17, which belong to the RPD3/HDA1 family, lack catalytic domains (Pandey et al., 2002). Thus, 14 HDACs in the RPD3/HDA1
plus HD2 families are targets for TSA and CAY, as described in Results. We constructed HDA-RNAi and HDT-RNAi libraries that corresponded to 10 genes in the RPD3/HDA1 family and four genes in the HD2 family, respectively. The DNA sequences of HDA9, HDA10, and HDA17, as annotated by The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org), are strongly homologous. We postulated that an RNAi construct specific for one of them would repress the expression of all three. Since the cDNA that encoded HDA17 was cloned so efficiently, we employed it instead of cDNA for HDA9 in the HDA-RNAi library. We used a partial genomic fragment for HDA7 since we were unable to clone the cognate cDNA. Each cDNA encoding the translation unit for an HDAC in the RPD3/HDA1 family, with the exception of HDA7, or for an HDAC in the HD2 family, as annotated by TAIR, was amplified by RT-PCR with appropriate primers (see Supplemental Table 3 online) and cloned into pENTR-D-TOPO (Invitrogen). Each construct was subcloned into the binary vector pYU501 (see Supplemental Methods online) for RNAi and introduced into Agrobacterium tumefaciens strain GV3101. After introduction of each plasmid into Agrobacterium, we transformed as2-1 35S:AS2-GR plants by the floral dip method.

Identification of the Relevant HDACs Using an RNAi Library

Transgenic plants carrying the RNAi library were screened on MS medium, without sucrose, that contained 3 mg/L thiamine, 5 mg/L nicotinic acid, 0.5 mg/L pyridoxine, 0.5 g/L MES-KOH, pH 5.7, 0.5% Gellan gum (Wako), and 15 μg/mL hygromycin B (Wako). The screenings were performed by reference to phenotype as described in Results. The introduced T-DNAs were identified by PCR using the appropriate vector primers (Rv59 [5’-CAGGAAACAGCTATGACCATGATTAC-3’] for HDT1, HDT2, and HDT4; and pU104 [5’-CTATCTTGCACAGGAACCTTCC-3’]) for HD3) and the primer specific to each HDT gene (pU608 for HD1; pU510 for HD2; pU512 for HD3; and pU514 for HD4). To confirm the nature of the T-DNA specific for RNAi of HD3, we performed nested PCR using pU803 (5’-GGGAGAGGATAAAGCAATGT-3’) and pU804 (5’-CAGGCTTCTTTGGGGTTTCCT-3’). The sequences of primers that are not given here can be found in Supplemental Table 2 online.

RT-PCR

RT-PCR was performed as described previously (Semiarti et al., 2001). The primers used for RT-PCR were as follows: for α-tubulin (TUBA; as a control), pU51 and pU52 (Semiarti et al., 2001); for HD1/H2A, pU723 (5’-ACATTTCCACGCTCTCAATAACCCCT-3’) and pU508 (see Supplemental Table 3 online); for HD2/H2B, pU771 (5’-CCTCTCTCTCTCTTCTGATGACACAACA-3’) and pU772 (5’-CCAAAAGCCTCTCTTCAGGATCCACACCC-3’); for HPT1, HPT1 (5’-AGGCTGAACCCGCCGAGCCT-3’) and HPT2 (5’-GACCCACGAGATGGTCGGG-3’); for HD3/H2C and HD4/H2D, the primers used to clone the cDNA for each HDT (see Supplemental Table 2 online); for PHB, pU579 (5’-ATGATGATGATTCCTTGGATCCA-3’); and pU580 (5’-GGGCCTCTCTGCTATAAGGAGTGGTCG-3’; for FL, pU221 (5’-AGGCATTATTTAGTATGGTGTCTG-3’) and pU222 (5’-AATAGAGTACACCAAGCTGAGG-3’); for pri-miR165, pU663 (5’-CATCATTTATCATACAAACCACCATCATAC-3’) and pU664 (5’-GGGGGAGGAGGACCTGCGCA-3’); and for pri-miR166, 6F and 7R (Jurecz et al., 2004).

Localization of Proteins

Synthesis of AS2-YFP, encoded by ER:AS2-YFP, was induced by treatment of appropriate transgenic plants with 5 μM estradiol for 20 min 1 d prior to observations. Fluorescence due to GFP and YFP in leaf epidermal cells was observed by confocal laser scanning microscopy (LSM 510 META; Carl Zeiss).

Note

Plasmids were constructed by standard techniques. Details are provided in Supplemental Methods online.

While RU acts antagonistically against the endogenous glucocorticoid in mammals, RU is used only as a weak agonist in this study because no GR is encoded in the Arabidopsis genome.

For screening of HDACs using the RNAi-library, we employed as2-1 35S:AS2-GR plants as a host instead of as2-1 mutants since these plants would also allow us to screen plants by reference to the suppression of overproduction of AS2-GR. However, no such screening was performed since we obtained transformants with a strong phenotype similar to that of as2-1 35S:AS2-GR plants, which resembled that of as2 mutant plants treated with inhibitors of HDACs.

Accession Numbers

GenBank/EMBL accession numbers and Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: HDT1/H2A (AT3G44750, NM_114344); HDT2/H2B (AT5G22650, NM_122171); AS1 (AT2G37630), NM_129319; and AS2 (AT1G65620), AB080802, NM_105235.

Supplemental Data

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

Supplemental Methods. Construction of Plasmids

Supplemental Figure 1. Acetylation Levels of Histone H4 Were Not Affected in as1 and as2 Mutants.

Supplemental Figure 2. Patterns of Expression of Reporter Genes from a Second 35S:erG3FP:c165 Transgenic Line and a 35S:GUS:c165 Plant.

Supplemental Figure 3. AS2-GFP-GR Fusion Proteins Were Localized in Nuclei of BY-2 Cells Treated with Dex.

Supplemental Figure 4. The 35S:AS2-GR Transgene Can Restore a Flat and Symmetric Leaf Shape in as2-1 Mutants in the Presence of Dex.

Supplemental Figure 5. Ectopic Overproduction of AS2-GR Reduces the Accumulation of miR165/166 and miR172.

Supplemental Figure 6. Repressive Accumulation of miR165/166 in 35S:AS2-GR Plants after Treatment with RU.

Supplemental Figure 7. Subnuclear Localization of GFP-AS1 and YFP-HDT1 Proteins.

Supplemental Table 1. Association between the Presence of Filamentous Leaves and the as2 Allele, HDT1, and HDT2.

Supplemental Table 2. Plant Materials and Chemicals Used in This Study.

Supplemental Table 3. Primers Used for Cloning the cDNAs for HDAC-Specific RNAi Libraries.

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Histone Deacetylases and ASYMMETRIC LEAVES2 Are Involved in the Establishment of Polarity in Leaves of *Arabidopsis*

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