HISTONE DEACETYLASE6-Defective Mutants Show Increased Expression and Acetylation of *ENHANCER OF TRIPTYCHON AND CAPRICE1* and *GLABRA2* with Small But Significant Effects on Root Epidermis Cellular Pattern[1]

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Cellular patterning in the Arabidopsis (*Arabidopsis thaliana*) root epidermis is dependent on positional information, the transmission of which involves histone acetylation. Here, we report that *HISTONE DEACETYLASE6* (*HDA6*) has significant effects on this cellular patterning. Mutation of *HDA6* led to ectopic hair cells in the nonhair positions of root epidermis in Arabidopsis, based on an analysis of paraffin sections stained with Toluidine Blue. While HDA6 was present throughout the root tip, epidermis-specific complementation with *HDA6* could rescue the *hda6* phenotype. Both transcript levels and expression patterns of *ENHANCER OF TRIPTYCHON AND CAPRICE1* (*ETC1*) and *GLABRA2* (*GL2*) in the root tip were affected in *hda6*. Consistent with these changes in expression, HDA6 directly bound to the promoter regions of *ETC1* and *GL2*, and acetylation of histone H3 on these promoter regions and acetylation of histone H4 on the *ETC1* promoter region was increased in the *hda6* mutant. Taken together, these results indicate that HDA6 affects the cellular patterning of Arabidopsis root epidermis through altering the histone acetylation status of *ETC1* and *GL2* promoters and thereby affects the expression of these two components of the core transcription factor network determining epidermal cell fates. Our findings thus provide new insights into the role of histone acetylation in root epidermis cell patterning.

Pattern formation is an important event during the morphogenesis of a multicellular organism. In Arabidopsis (*Arabidopsis thaliana*), root epidermis is a well-established model system for studying pattern formation in plant development (Schiefelbein, 2003; Schiefelbein et al., 2009, 2014; Grebe, 2012). The root epidermis comprises hair cells and nonhair cells. These two types of epidermal cells have different cytoplasmic characteristics, and their fates are determined in a position-dependent manner: epidermal cells overlying two cortical cells (the H position) adopt the hair cell fate, while epidermal cells located over a single cortical cell (the N position) adopt the nonhair cell fate (Dolan et al., 1993; Galway et al., 1994; Berger et al., 1998).

The position-dependent cellular patterning of Arabidopsis root epidermis is regulated mainly by a system consisting of at least three levels. The first level is a GLABRA2 (GL2)-centered transcription factor network including three types of proteins: the Myb domain proteins WEREWOLF (WER), CAPRICE (CPC), TRIPTYCHON (TRY), and ENHANCER OF TRIPTYCHON AND CAPRICE1 (ETC1; Wada et al., 1997; Lee and Schiefelbein, 1999; Schellmann et al., 2002; Kirik et al., 2004); the basic helix-loop-helix proteins GL3 and ENHANCER OF GALBRA3 (EGL3; Bernhardt et al., 2003, 2005); and a WD-repeat protein, TRANSPARENT TESTA GLABRA (TTG; Galway et al., 1994; Walker et al., 1999; Supplemental Fig. S1). The genes encoding these proteins are referred to as pattern genes and determine the fates of epidermal cells in the N and H positions. Additional components functioning at this level include MYB23 (Kang et al., 2009), GEM (for *GL2* expression modulator; Caro et al., 2007), MYC1 (Bruex et al., 2012), Zinc Finger Protein5 (An et al., 2012), and ADENOSINEDIMETHYL TRANSFERASE1A (Wieckowski and Schiefelbein, 2012). The second level of the root epidermis cellular patterning system is sensor pathways consisting of membrane-localized receptor-like kinases, such as SCRAMBLED (SCM; Kwak et al., 2005; Kwak and Schiefelbein, 2007, 2008) and BRASSINOSTEROID INSENSITIVE1 (Kuppusamy et al., 2009). These factors seem to sense and interpret the positional

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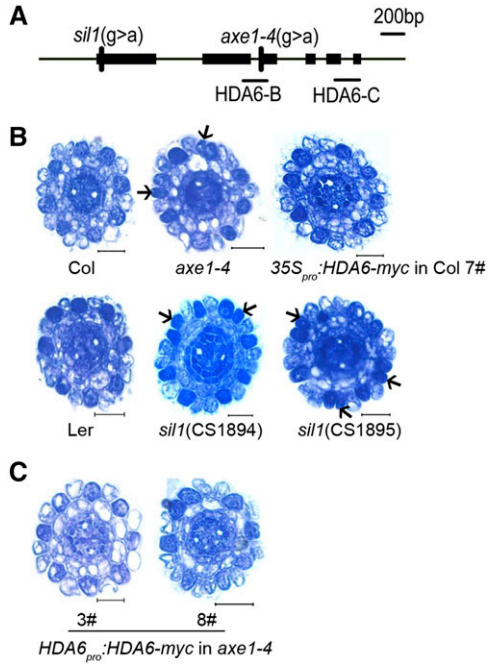

Figure 1. The *hda6* mutant displays ectopic development of hair cells at the N position in the root epidermis. A, Schematic representation of *hda6* point mutations. There are two *sil1* mutant lines, CS1894 and CS1895, both with a point mutation 46 bp after the start codon that results in the replacement of Gly-16 by Arg. The *axe1-4* mutant has a base substitution at the second intron-exon junction (1,492 bp downstream of the start codon). The short black lines indicate regions selected for quantitative reverse transcription (qRT)-PCR. B, Cross sections of root tips of Col, *axe1-4*, and overexpression line (top row, left to right), and L*er*, *sil1* (CS1894), and *sil1* (CS1895; bottom row, left to right). Arrows indicate ectopically developing hair cells. Bars = 20 μm. C, Cross sections of root tips of *hda6* complementation lines harboring a genomic *HDA6* DNA fragment. Bars = 20 μm.

information. The third level of the system concerns the source of the positional information; at this level, JACKDAW functions in the cortical cells to affect epidermal cell patterning (Hassan et al., 2010). However, little is known about how the positional information is transmitted from cortical cells to the epidermal cells.

In our previous work, we found that treatment with trichostatin A (an inhibitor of histone deacetylases) can alter cellular patterning in the Arabidopsis root epidermis by affecting the histone acetylation status at promoters of the pattern genes (Xu et al., 2005). HISTONE DEACETYLASE18 (HDA18), a member of the HDAC (for histone deacetylase) family, affects epidermal cell patterning by regulating the transcription of a group of kinase genes through histone acetylation (Liu et al., 2013a). In a phenotype screen of single mutants from the Arabidopsis HDAC family, we found that the single mutant of *HDA6* also displayed altered cellular patterning of root epidermis. HDA6 is a class I reduced potassium dependency3-like HDAC (Pandey et al., 2002) and has been reported to have HDAC enzyme activity (Earley et al., 2006). HDA6 is localized in the nucleus, supporting its involvement in transcriptional regulation (Earley et al., 2006; Wu et al., 2008). HDA6 is involved in many biological processes, including gene silencing (Murfett et al., 2001; Probst et al., 2004; Earley et al., 2006, 2010; Liu et al., 2012; Pontvianne et al., 2013), RNA-directed DNA methylation (Aufsatz et al., 2002, 2007), jasmonic acid response (Devoto et al., 2002; Wu et al., 2008; Zhu et al., 2011), and flowering (Wu et al., 2008; Yu et al., 2011).

Here, we demonstrate the involvement of HDA6 in affecting the cellular patterning in Arabidopsis root epidermis via various genetic approaches. We found evidence that HDA6 directly binds to the promoter regions of pattern genes *ETC1* and *GL2*, which are involved in regulating epidermal root cell identity, and loss of HDA6 activity causes increased acetylation and expression of *ETC1* and *GL2*, resulting in altered cellular patterning. In addition, we found subtle differences between the effects of HDA6 on *ETC1* and *GL2*. Taken together with the role of HDA18, we conclude that histone acetylation plays indispensable roles in various aspects of the regulating system in determining the cellular pattern of Arabidopsis root epidermis.

Table I. *Cellular patterning in the root epidermis of hda6 mutants and transgenic plants*

At least 12 7-d-old seedlings were examined for each plant line. Values indicate means ± SD.

Genotype	H Position		N Position	
	Developing Hair Cell	Developing Nonhair Cell	Developing Hair Cell	Developing Nonhair Cell
	%			
Col	99.1 ± 1.4	0.9 ± 1.4	2.1 ± 2.9	97.9 ± 2.9
axe1-4	100.0 ± 0.0	0.0 ± 0.0	13.4 ± 6.7[a]	86.6 ± 6.7[a]
35S$_{pro}$:HDA6-myc in Col	96.7 ± 5.2	3.3 ± 5.2	2.6 ± 3.9	97.4 ± 3.9
HDA6$_{pro}$:HDA6-myc in *axe1-4* 3#	98.8 ± 2.2	1.2 ± 2.2	2.6 ± 4.0	97.4 ± 4.0
HDA6$_{pro}$:HDA6-myc in *axe1-4* 8#	99.4 ± 1.0	0.6 ± 1.0	2.8 ± 4.2	97.2 ± 4.2
L*er*	98.6 ± 2.5	1.4 ± 2.5	2.8 ± 4.2	97.2 ± 4.2
sil1 (CS1894)	98.9 ± 2.2	1.1 ± 2.2	12.3 ± 5.1[a]	87.7 ± 5.1[a]
sil1 (CS1895)	100.0 ± 0.0	0.0 ± 0.0	17.2 ± 5.6[a]	82.8 ± 5.6[a]

[a]Differs significantly from the wild type ($P < 0.01$; Student's *t* test).

RESULTS

HDA6 Is Required for Proper Cellular Patterning of Arabidopsis Root Epidermis

To test for *HDA6* involvement in the cellular patterning of Arabidopsis root epidermis, we analyzed the available *hda6* mutants, namely *auxin gene expression mutants1-4* (*axe1-4*; Columbia [Col] background bearing $DR5_{pro}$:*GUS*, a base substitution [G→A] at position 1,492 bp downstream of the ATG, at the second intron-exon junction; Murfett et al., 2001) and *silencing1* (*sil1;* Landsberg *erecta* [L*er*] background, CS1894 and CS1895, each having a G→A point mutation, 46 bp after the start codon and resulting in the replacement of Gly-16 by Arg; Probst et al., 2004). The expression of *HDA6* in the root tip was reduced about 80% in the *axe1-4* mutant compared with Col and was reduced only 10% to 15% in the *sil1* mutant compared with L*er* (Fig. 1A; Supplemental Fig. S2, A and B). Both *axe1-4* and *sil1* showed that the densely stained N-position cells were increased from around 2% to 3% in the wild type to around 12% to 17% in the mutant, indicative of ectopically developing hair cells at the N position in the epidermis (Fig. 1B; Table I).

To examine whether the altered cellular pattern in Arabidopsis was indeed caused by *HDA6* mutation, we constructed complementation lines by transferring the genomic *HDA6* sequence with a 9× Myc tag under the control of its native promoter into the *axe1-4* mutant (Supplemental Fig. S3, A and C). Among the various lines, line 3 had an expression level of *HDA6* similar to Col, while line 8 had nearly 8-fold increased *HDA6* expression in the root tip (Supplemental Fig. S3B). Therefore, line 3 was used as a complementation line and line 8 as an overexpression line. Analysis of paraffin sections revealed that the altered cellular pattern in the root epidermis of *axe1-4* was rescued in both lines (Fig. 1C; Table I), indicating that, on the one hand, *HDA6* was indeed involved in root epidermis cellular patterning, and on the other hand, overexpression of *HDA6* does not interfere with the cellular patterning phenotype (Fig. 1B; Table I; Supplemental Fig. S2A). Further confirming the lack of effect of overexpression, cellular patterning was not altered in the root epidermis of a $35S_{pro}$:*HDA6-myc* overexpression line in the Col background (Fig. 1B; Table I; Supplemental Fig. S2A).

HDA6 Is Expressed throughout the Arabidopsis Root Tip But Affects the Cellular Patterning in Epidermis

According to root gene expression data provided by AREX LITE (http://www.arexdb.org/; Brady et al., 2007), *HDA6* is expressed in all root tip cells (Supplemental Fig. S4, B and C). To confirm the expression pattern of *HDA6* at both the RNA and protein levels in Arabidopsis root tips, we constructed transgenic lines $HDA6_{pro}$:*EGFP* (for *enhanced GFP*) and $HDA6_{pro}$:*HDA6-EGFP* in the Col background (Supplemental Fig. S4A). EGFP signal was detected in all tissues of the root tip, including N-position and H-position cells of the root epidermis of both lines (Fig. 2). These results indicate that both *HDA6* RNA and HDA6 protein are present ubiquitously in Arabidopsis root tips, which is consistent with the previous information.

To clarify whether the effects of HDA6 on the cellular patterning is epidermis specific, we expressed HDA6 protein exclusively in root epidermis using a 2.5-kb *WER* promoter (Lee and Schiefelbein, 1999) in the *axe1-4* and CS1895 backgrounds (Supplemental Fig. S5). As shown in Figure 3, A to D, HDA6 was specifically expressed in root epidermis in the WER_{pro}:*HDA6-EGFP axe1-4* and CS1895 lines. The altered cellular patterning of the *hda6* mutants was successfully rescued in these transgenic lines (Fig. 3E), indicating that HDA6's effects on cellular patterning are epidermis specific.

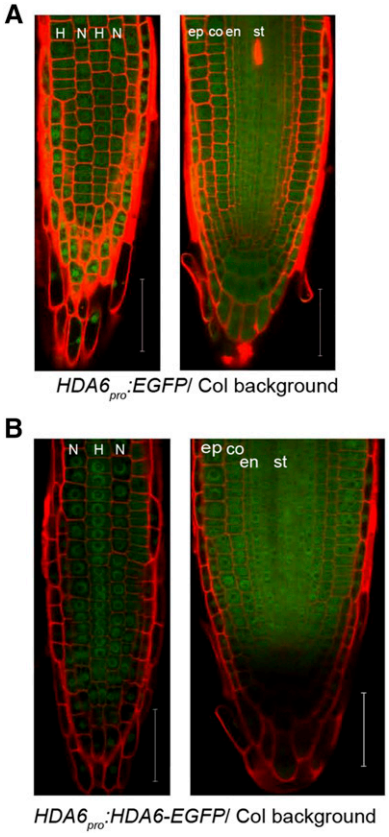

Figure 2. HDA6 localizes ubiquitously in Arabidopsis root tip. A, Confocal laser scanning microscope (CLSM) images of 7-d-old root tip of $HDA6_{pro}$:*EGFP* reporting the location of *HDA6* expression: epidermal view (left) and median view (right). Bars = 50 μm. B, CLSM images of 7-d-old root tip of $HDA6_{pro}$:*HDA6-EGFP* showing HDA6 protein localization: epidermal view (left) and median view (right). Bars = 50 μm. In all images, N indicates nonhair cell position and H indicates hair cell position. co, Cortex; en, endodermis; ep, epidermis; st, stele. Red areas are propidium iodide (PI) signals and green areas are GFP signals.

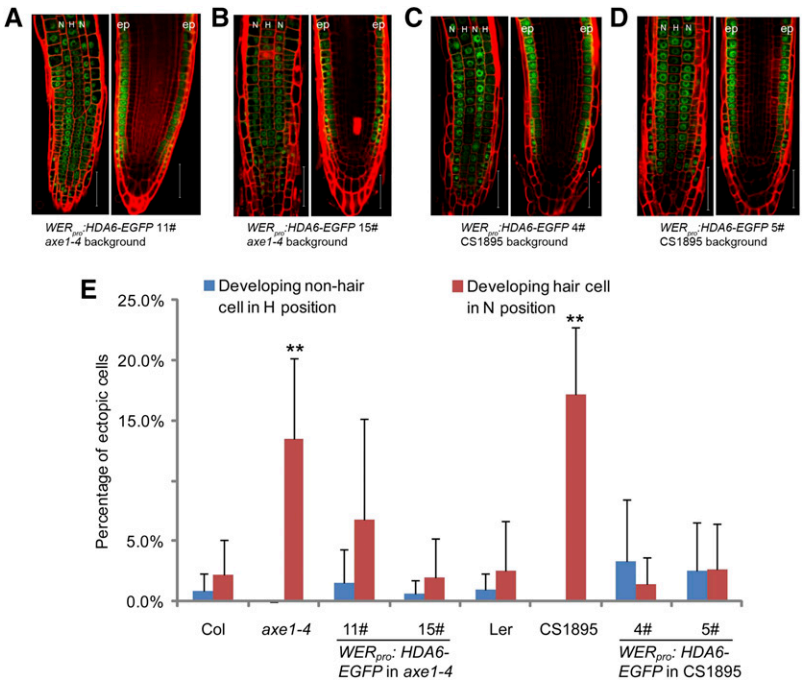

Figure 3. Epidermis-specific expression of *HDA6* can restore the altered root epidermal cellular pattern of the *hda6* mutant. A to D, Seven-day-old roots of WER_{pro}:*HDA6-EGFP* transgenic plants were analyzed for HDA6-EGFP localization in the root tip. Note that A and B are *axe1-4* background and C and D are CS1895 background. Epidermal view is on the left and median view is on the right. N indicates nonhair cell position and H indicates hair cell position. ep, Epidermis. Bars = 50 μm. E, Quantification of the root epidermal cellular pattern of transgenic plants, showing frequencies of ectopically developing non-hair cells (blue bars) and ectopically developing hair cells (red bars). At least 12 root tips were analyzed for each line. The mean and SD are indicated for each line. **, $P < 0.01$, Student's *t* test.

HDA6 Affects Both the Levels and Patterns of *ETC1* and *GL2* Expression

Next, we asked whether HDA6 influences cellular patterning through affecting pattern genes. We checked the expression of known pattern genes in the root tip of *hda6* mutants (*axe1-4* and CS1895), of the above-mentioned complementation line, $HDA6_{pro}$:*HDA6-myc*/*axe1-4* 3#, and of overexpression line $35S_{pro}$:*HDA6-myc*/Col 7#. As shown in Figure 4A, expression levels of *WER*, *TRY*, *GL3*, and *EGL3* were decreased in *axe1-4* but were rescued in the complementation line. Expression of *CPC*, *ETC1*, *TTG*, *GL2*, *SCM*, and *GEM* did not show obvious changes in *axe1-4*. However, the expression of *TTG* and *SCM* increased unexpectedly in the complementation line (Fig. 4A). In CS1895, the expression levels of only *TRY* and *GL2* were slightly reduced (Supplemental Fig. S6). In the overexpression line, the expression levels of *CPC* and *TRY* were decreased while those of *EGL3* and *GL2* were slightly increased (Fig. 4A). These results indicate that altered expression of HDA6 indeed affects the expression of pattern genes.

To identify the potential targets of HDA6, we analyzed HDA6's effect on the location of the expression of pattern genes such as *GL2*, *WER*, *CPC*, and *ETC1* by introducing $GL2_{pro}$:*GFP*, WER_{pro}:*GFP*, CPC_{pro}:*GUS*, and $ETC1_{pro}$:*GUS* into *hda6* mutants. We found that $GL2_{pro}$:*GFP* was ectopically expressed in cells at the H position in the root epidermis of *hda6* mutants (Fig. 4B). $ETC1_{pro}$:*GUS* was also ectopically expressed in a few H-position cells in root epidermis of *hda6* (Fig. 4D). By contrast, there were no alterations in the signals detected for WER_{pro}:*GFP* and CPC_{pro}:*GUS* in *hda6* (Fig. 4, C and E). These results suggested that *GL2* and *ETC1* might be targets of HDA6.

As mentioned above, we did not detect any changes in the expression level of *GL2* and *ETC1* in *hda6* (Fig. 4A; Supplemental Fig. S6). It is possible that *GL2* and *ETC1* were ectopically expressed in only a few cells of the *hda6* mutant (Fig. 4, B and D) and that any changes in *GL2* and *ETC1* expression were lost in the overall expression analyzed using the entire root tip. We reasoned that if we could reduce the expression of *GL2* and *ETC1* in the root tip, we might uncover an effect of HDA6 on the expression level of *GL2* and *ETC1* in the epidermis, for instance. According to the current model (Supplemental Fig. S1), *ETC1* and *GL2* are positively regulated by the WER-GL3/EGL3-TTG complex (Bernhardt et al., 2003, 2005; Morohashi et al., 2007). Accordingly, we crossed *axe1-4* into a *gl3/egl3* double mutant, which has low expression of *GL2* and *ETC1* (Fig. 4F). Compared with *gl3/egl3*, the expression levels of *GL2* and *ETC1* in the root of the *gl3/egl3/axe1-4* triple mutant were significantly increased, while the expression of *WER*, *CPC*, and *TTG* remained unchanged (Fig. 4F). These results indicate that HDA6 can affect the transcript levels of *GL2* and *ETC1* and may be antagonistic to GL3/EGL3.

Taken together, the above data suggest that *GL2* and *ETC1* could be target genes of HDA6 to regulate the cellular patterning of Arabidopsis root epidermis.

HDA6 Binds to Promoters of *ETC1* and *GL2* and Affects the Histone Acetylation Status of the Bound Regions

To examine whether HDA6 directly affects *GL2* and *ETC1*, we performed chromatin immunoprecipitation (ChIP) assays using $35S_{pro}$:*HDA6-myc* transgenic plants with commercial Myc antibody (Supplemental Fig. S7).

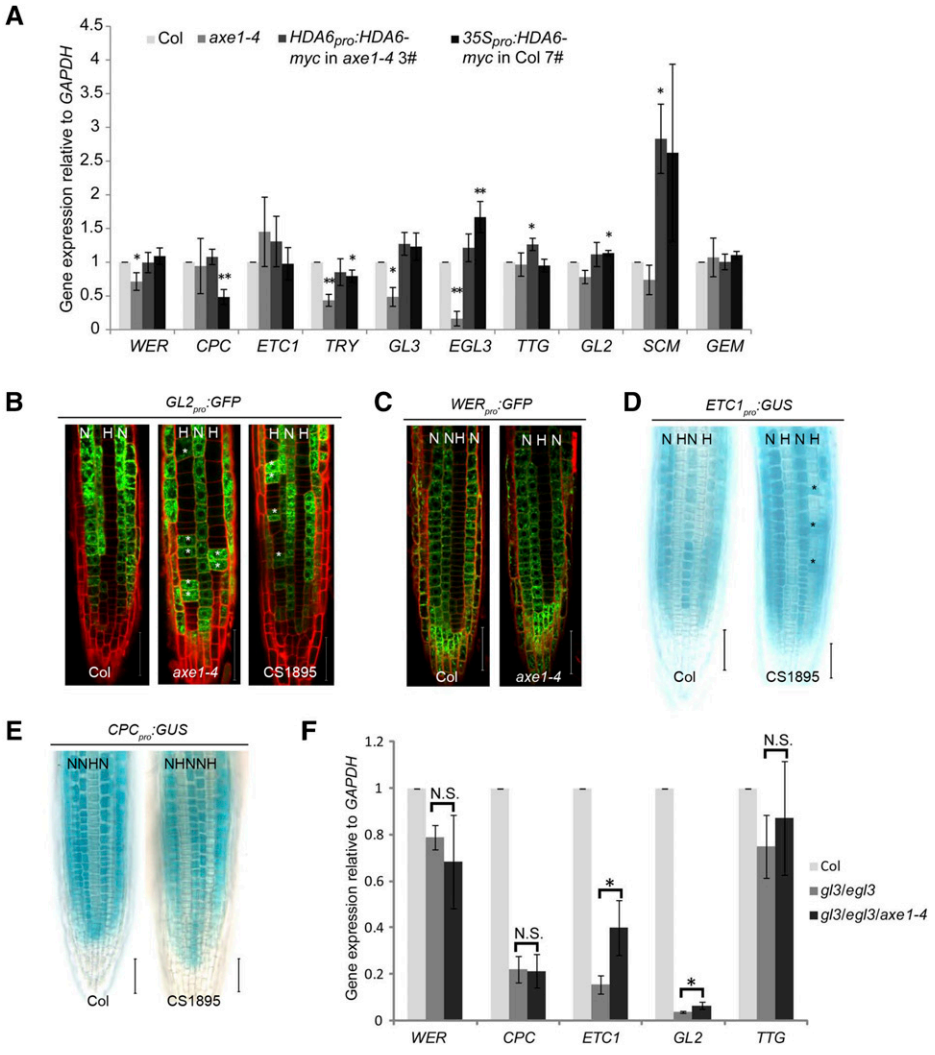

Figure 4. HDA6 affects the expression level and pattern of *ETC1* and *GL2*. A, Expression levels of pattern genes were analyzed in root tips of *axe1-4*, *HDA6* complementation line, and *HDA6* overexpression line compared with Col. Error bars represent SD values from at least three biological replicates each consisting of three technical replicates. **, $P < 0.01$ and *, $P < 0.05$, Student's *t* test. B, Root epidermal view of 7-d-old seedlings bearing the $GL2_{pro}$:*GFP* reporter gene. Bars = 50 μm. C, Root epidermal view of 7-d-old seedlings bearing the WER_{pro}:*GFP* reporter gene. Bars = 50 μm. D, Seven-day-old seedlings bearing the $ETC1_{pro}$:*GUS* reporter gene were stained for GUS activity. Bars = 50 μm. E, Seven-day-old seedlings bearing the CPC_{pro}:*GUS* reporter gene were stained for GUS activity. Bars = 50 μm. In B to E, N indicates nonhair cell position, H indicates hair cell position, and asterisks indicate ectopic reporter gene-expressing cells. F, *ETC1* and *GL2* expression levels are significantly increased in the root tip of *gl3/egl3/axe1-4* compared with *gl3/egl3*. Error bars represent SD values from at least three biological replicates each consisting of three technical replicates. *, $P < 0.05$, Student's *t* test; N.S., no significance.

Regions on *ETC1* and *GL2* promoters for ChIP-PCR were selected randomly (Fig. 5A). Enrichments of the selected sequences by Myc antibody (Fig. 5B) demonstrated that HDA6 directly binds to some of the selected regions in the *ETC1* and *GL2* promoters, and not to those in *EGL3*, *CPC*, and *TUBULIN* promoters, consistent with the effects of the *HDA6* gene on the respective pattern genes described above.

Since HDA6 has been reported to have histone deacetylase enzyme activity (Earley et al., 2006), we further monitored whether histone acetylation status at the HDA6-binding regions was affected by the *hda6* mutation by performing a ChIP assay with acetylated histone antibodies. Both H3 and H4 acetylation levels at the *ETC1* promoter regions bound by HDA6 were significantly increased in *axe1-4* (Figs. 5B and 6). However, while H3 acetylation levels at three of the four HDA6-binding sites of *GL2* promoter regions were increased in *axe1-4* (Figs. 5B and 6), no H4 acetylation increase was detected at the HDA6-binding sites of the *GL2* promoter in *axe1-4* (Figs. 5B and 6). These data indicate that HDA6 likely affects the expression of *ETC1* and *GL2* through affecting the histone acetylation status of their promoters, but the detailed mechanism regarding histone types and binding sites is different for each gene.

HDA6 Is Genetically Upstream of *ETC1* in the Cellular Patterning of Root Epidermis

It has been reported that *ETC1* overexpression causes excessive root hair production, although the *etc1* single mutant has no significant root hair phenotype (Kirik et al., 2004). Consistent with that report, we found that *etc1* had normal differential staining of epidermal cells (Fig. 7A), while *ETC1* overexpression caused almost all of the N-position epidermal cells to be densely stained like the H-position epidermal cells, indicating that the N-position cells were transformed into developing hair cells (Supplemental Fig. S8). To further clarify the genetically regulatory relationship between *HDA6* and *ETC1*, we constructed *hda6/etc1* double mutants. Similar to the phenotype of *etc1*, both *axe1-4/etc1* and CS1895/*etc1* double mutants had normal differential staining of epidermal cells (Fig. 7,

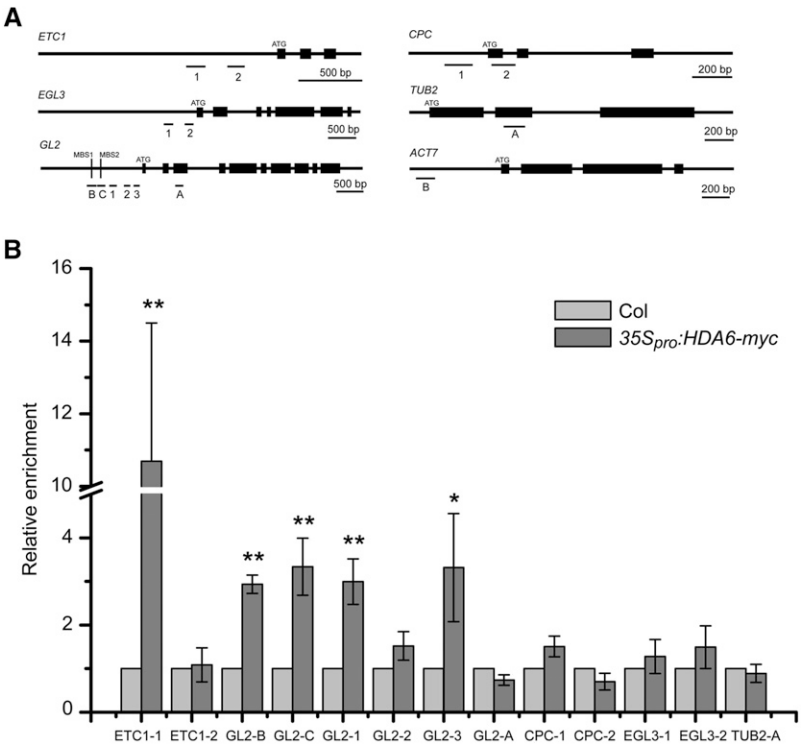

Figure 5. HDA6 binds to the promoter regions of *ETC1* and *GL2*. A, Schematic representation of selected regions for ChIP-PCR analyses. B, Enrichment of HDA6 at the promoter regions of *ETC1* and *GL2* was tested by ChIP-PCR. Transgenic plants bearing the *35S$_{pro}$:HDA6-myc* construct were subjected to ChIP assays with an anti-myc antibody. Col plants were used as negative controls. *ACTIN7* and *TUBULIN2* (*TUB2*) were used as an internal control for normalization. Error bars represent SD values from at least three biological replicates each consisting of three technical replicates. **, $P < 0.01$ and *, $P < 0.05$, Student's *t* test.

A and B). This suggests that *ETC1* is genetically epistatic to *HDA6* in affecting the cellular pattern of Arabidopsis root epidermis, consistent with the idea that HDA6 affects the cellular pattern phenotype through directly binding and acetylating histones at *ETC1* promoter regions.

To further demonstrate that HDA6 is upstream of *ETC1*, we introduced *WER* promoter-driven *HDA6* into the *hda6* mutant (CS1895) containing the *ETC1$_{pro}$:GUS* marker (Fig. 7C) and found that epidermis-specific expression of *HDA6* could rescue the altered expression pattern of *ETC1* in the *hda6* mutant.

HDA6 Can Directly Affect *GL2* Expression Independently of Its Effect on *ETC1*

According to the current model, *ETC1* can function redundantly with *CPC* and *TRY* to repress *GL2* expression (Grebe, 2012; Schiefelbein et al., 2014). Our data showed that while HDA6 directly affects *ETC1* by binding its promoter and affecting the histone acetylation status at the binding region, HDA6 can also directly bind the *GL2* promoter and affect H3 acetylation at its binding sites (Fig. 6). This suggested that HDA6 may be able to influence *GL2* expression independently of *ETC1*. We began by introducing a *GL2* promoter reporter into the *ETC1* overexpression line. As predicted by the model described above, the *GL2* promoter activity was severely suppressed by overexpression of *ETC1* (Fig. 8A), while *GL2* expression was a little increased in the *axe1-4* root, in which *ETC1* expression was increased (Fig. 4). The observation that *GL2* expression in *axe1-4* is not suppressed by increased *ETC1* expression in the same background suggests that the effect of HDA6 on *GL2* may be independent of *ETC1*. To test this, we examined *GL2* promoter activity in both *etc1* single mutant and *axe1-4/etc1* double mutant backgrounds. We found that while the *GL2* expression pattern was not affected in the *etc1* single mutant compared with the wild type (Fig. 4B), it was significantly altered in the *axe1-4/etc1* double mutant (Fig. 8, B and C), similar to that in the *axe1-4* single mutant (Fig. 4B). These genetic data suggest that while overexpression of *ETC1* indeed affects *GL2* expression, HDA6 can directly affect *GL2* expression independent of its effect on *ETC1*.

To further demonstrate the effects of HDA6 on *GL2* expression, we also introduced *WER* promoter-driven *HDA6* into the *hda6* mutant (CS1895) containing the *GL2$_{pro}$:GUS* marker (Fig. 8D), showing that the epidermis-specific expression of *HDA6* could rescue the altered expression pattern of *GL2* in the *hda6* mutant.

The effect of HDA6 upon *GL2* could further be supported by observation of the inhibition of root hair initiation in the *hda6* mutant. GL2 plays a key role in root hair initiation and elongation (Di Cristina et al., 1996; Masucci et al., 1996). In the *hda6* mutant, we found that while more developing hair cells, based on deep staining, were found in the N position by sections of root tips (Fig. 1), fewer root hairs were observed (Fig. 9, A and B). It is not clear how GL2 regulates root hair initiation and elongation. However, some genes related to root hair initiation, such as *PHOSPHOLIPASE D ZETA1* (*PLDζ1*), *CELLULOSE SYNTHASE5* (*CESA5*), *XYLOGLUCAN ENDOTRANSGLUCOSYLASE17*, and *ROOT HAIR DEFECT6* (*RHD6*), are known to be regulated by GL2 (Ohashi et al., 2003; Tominaga-Wada et al., 2009;

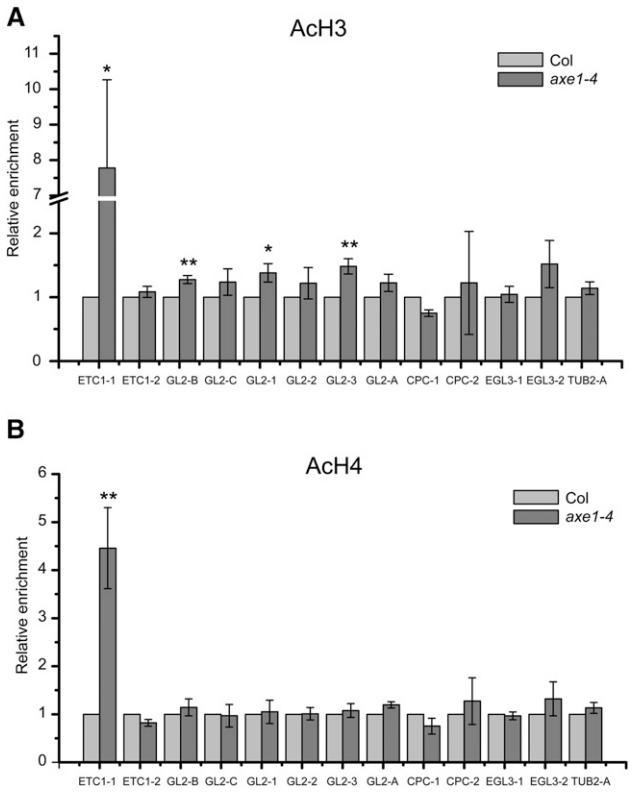

Figure 6. HDA6 affects histone acetylation of the *ETC1* and *GL2* promoter regions. A, Histone H3 acetylation status at selected promoter regions of pattern genes in *axe1-4* was analyzed by ChIP assay using antibodies specific to acetylated histone H3K9K14 followed by quantitative PCR. *ACTIN7* was used as an internal control for normalization. Error bars represent SD values from at least three biological replicates each consisting of three technical replicates. **, $P < 0.01$ and *, $P < 0.05$, Student's *t* test. B, Histone H4 acetylation status at selected promoter regions of pattern genes in *axe1-4* was analyzed by ChIP assay using antibodies specific to acetylated histone H4K5K8K12K16. *ACTIN7* was used as an internal control for normalization. Error bars represent SD values from at least three biological replicates each consisting of three technical replicates. **, $P < 0.01$, Student's *t* test. TUB2, TUBULIN2.

Bruex et al., 2012; Schiefelbein et al., 2014). Therefore, we examined the expression of these genes downstream of *GL2*. We found that, consistent with the finding that HDA6 represses *GL2* expression (Fig. 4, B and F), GL2-repressed genes such as *PLDζ1*, *CESA5*, and *RHD6* were up-regulated in the *gl2* mutant and repressed in the *hda6* mutant, in which *GL2* expression is increased (Fig. 9C).

DISCUSSION

Previously, mainly based on the effects of trichostatin A, an inhibitor of histone deacetylase, we proposed that histone acetylation is involved in the regulation of cellular pattern formation in Arabidopsis root epidermis (Xu et al., 2005). Functional analysis of HDA18 showed that it directly binds and regulates kinase genes involved in cellular patterning (Liu et al., 2013a). Here, we report another mechanism by which *HDAC* genes affect cellular patterning; in this case, HDA6 affects the expression of *ETC1* and *GL2*, two transcription factors that are components of the network determining the cell fates at the N and H positions in root epidermis. Taken together with the previously reported role of GEM in cell fate determination through histone modification (Caro et al., 2007), it is clear that, while the involvement of HDACs in the cellular patterning of Arabidopsis root epidermis is confirmed, the detailed mechanisms are highly diversified for each member of the HDAC family. Considering the key roles of the GL2-centered transcription factor network in cellular patterning in Arabidopsis root epidermis, it seems that histone acetylation plays an important role in maintaining the

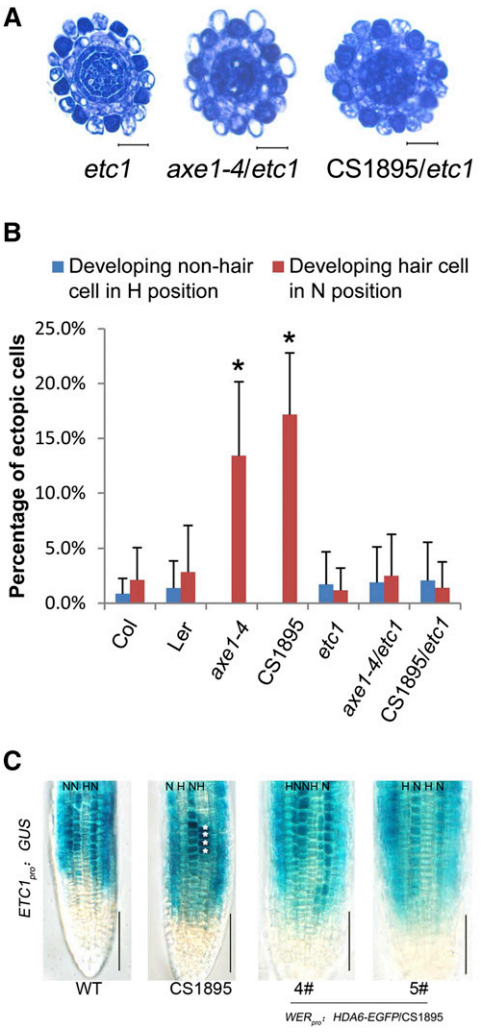

Figure 7. Phenotype analysis of the *hda6/etc1* double mutant. A, Cross sections of root tips of *etc1* and *hda6/etc1*. Bars = 20 μm. B, Quantification of the root epidermal cellular pattern of the *hda6/etc1* double mutant, showing frequencies of ectopic developing nonhair cells (blue bars) and ectopic developing hair cells (red bars). At least 12 root tips were analyzed for each line. The mean and SD are indicated for each line. *, $P < 0.05$, Student's *t* test. C, Seven-day-old seedlings bearing the $ETC1_{pro}$:*GUS* reporter gene were stained for GUS activity. WT, Wild type. Bars = 50 μm.

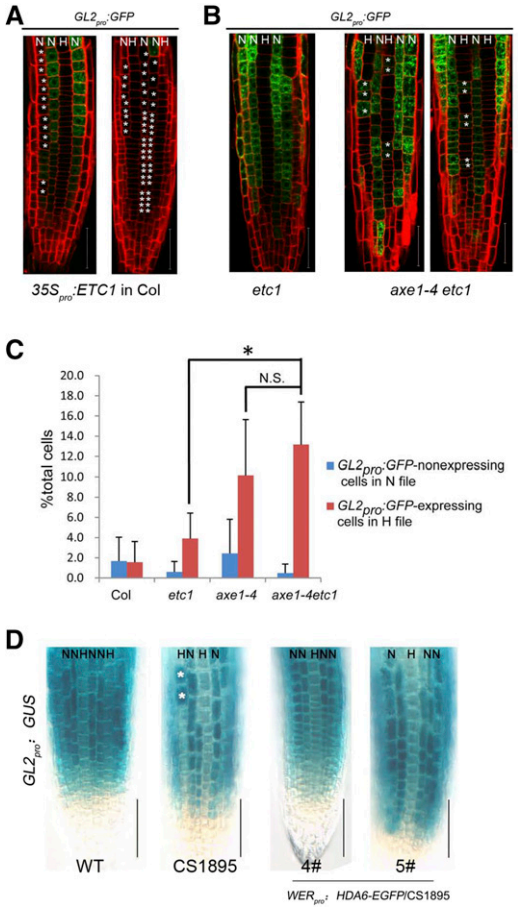

Figure 8. Analysis of *HDA6*'s effects on *GL2*. A, CLSM images of root tip of *ETC1* overexpression lines bearing the $GL2_{pro}$:*GFP* reporter gene. The asterisks indicate ectopic $GL2_{pro}$:*GFP*-nonexpressing cells in the N position. Bars = 50 μm. B, CLSM images of root tip of the *hda6/etc1* double mutant bearing the $GL2_{pro}$:*GFP* reporter gene. The asterisks indicate ectopic $GL2_{pro}$:*GFP*-expressing cells in the H positon. Bars = 50 μm. C, Quantification of the epidermal cell type pattern, showing percentages of ectopic $GL2_{pro}$:*GFP*-nonexpressing cells in the N position (blue bars) and ectopic $GL2_{pro}$:*GFP*-expressing cells in the H positon (red bars) for each line. *, $P < 0.05$, Student's *t* test; N.S., no significance. D, Seven-day-old seedlings bearing the $GL2_{pro}$:*GUS* reporter gene were stained for GUS activity. WT, Wild type. Bars = 50 μm.

robustness of the GL2-centered transcription factor network, through directly maintaining the proper expression levels and expression patterns of transcription factors such as *ETC1* and *GL2*, as for HDA6, and/or adjusting the transmission of positional information from cortical cells, as for HDA18.

HDA6 has been demonstrated to be involved in the regulation of many physiological processes in plants. It has been reported that HDA6 and HDA19 interact with various transcription factors and chromatin-remolding factors and play different roles, with a range of target genes in various developmental processes (Liu et al., 2014). Here, we demonstrated that, in the involvement in cellular patterning, HDA6 selectively affects the expression of *ETC1* and *GL2* but not other known pattern genes. Our results suggest that, even in this particular process, HDA6 plays different roles through different target genes. These characteristics bring up the question of how particular HDAC family members evolved to regulate the expression of some genes but not others. From this perspective, the cellular patterning of Arabidopsis root epidermis can serve as a good system in which to investigate how particular members of the HDAC family function upon various target genes and how these effects are integrated together.

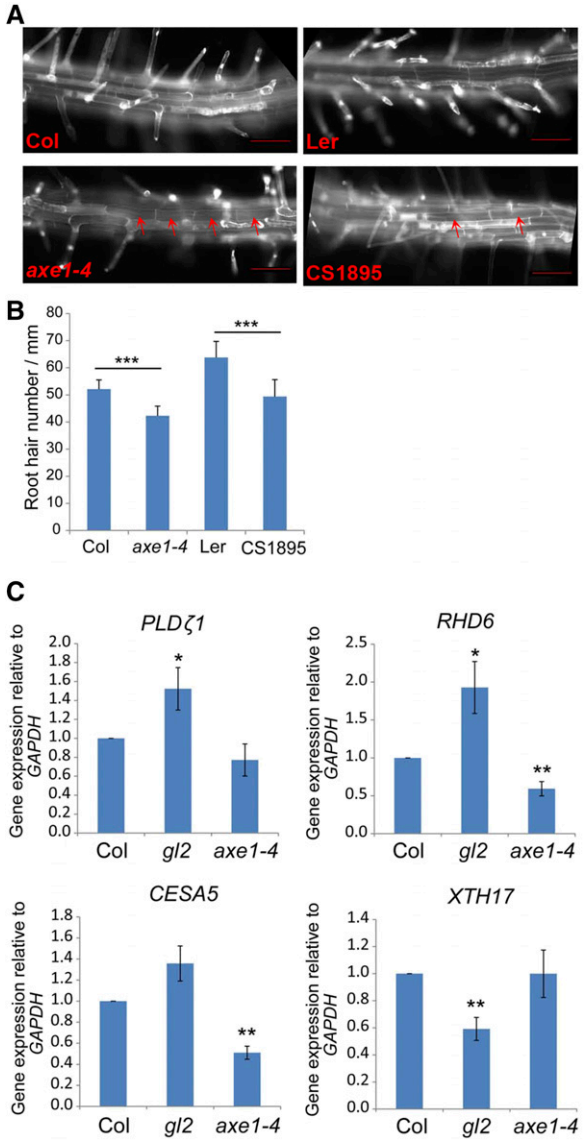

Figure 9. Root hair number was reduced in *hda6* roots. A, Ectopic nonroot hairs at the H position were observed in the mature zone of *hda6* root. Bars = 50 μm. B, Root hair density was reduced in *hda6* root. At least 20 roots were analyzed for each line. ***, $P < 0.001$, Student's *t* test. C, The expression of GL2 downstream genes was detected in *hda6*. **, $P < 0.01$ and *, $P < 0.05$, Student's *t* test.

It is worth noting that, compared with the phenotypes of the mutants of the pattern genes, such as *wer*, *cpc*, and *gl2*, the phenotypes of *hda6* in this work and *hda18* in previous work (Liu et al., 2013a) were not as prominent. One explanation could be that, in cellular patterning, the regulatory network of transcriptional factors are the key players and the histone modification of the transcription of the components comprising the network plays a role in enhancing the robustness of the regulatory network.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) *hda6* point mutants *axe1-4* and *sil1* (CS1894 and CS1895) were purchased from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org/). The following mutants and reporter lines were previously described: *etc1* and $ETC1_{pro}$:*GUS* (Kirik et al., 2004), WER_{pro}:*GFP* (Lee and Schiefelbein, 1999), $GL2_{pro}$:*GFP* (Lin and Schiefelbein, 2001), $EGL3_{pro}$:*GUS* (Zhang et al., 2003), and CPC_{pro}:*GUS* (Wada et al., 2002). Double and triple mutants were constructed by crossing. Reporter lines were introduced into mutants or transgenic plants by crossing. Homozygous lines were verified by molecular genotyping, phenotype analysis, and reporter gene expression. Seeds were sterilized and planted on 0.5× Murashige and Skoog medium solidified with 0.8% (m/v) agar. Plates were placed in the dark at 4°C for 2 d and then moved to 22°C with a period of 16 h of light/8 h of dark.

Construction of Plasmids and Transgenic Plants

For the $35S_{pro}$:*HDA6-myc* construct, the coding sequence of *HDA6* (without the stop codon) was first fused in frame to the 5′ end of nine tandem repeats encoding the MYC epitope, and then the fusion was cloned into the pDR vector containing the *CaMV35S* promoter. For the $HDA6_{pro}$:*HDA6-myc* construct, a 2,918-bp genomic fragment of *HDA6* was first fused in frame to the 5′ end of nine tandem repeats encoding the MYC epitope, and then the fusion was cloned into a modified pDR vector containing the 3′ untranslated region and intergenic sequence of *HDA6*. For the $HDA6_{pro}$:*EGFP* construct, a 524-bp region upstream of the *HDA6* start codon was introduced into the pEGAD-EGFP vector to replace the *CaMV35S* promoter. For the $HDA6_{pro}$:*HDA6-EGFP* construct, the same fragment as used for the $HDA6_{pro}$:*HDA6-myc* construct was introduced into the pEGAD-EGFP vector to replace the *CaMV35S* promoter and fused in frame to the 5′ end of the sequence encoding the EGFP epitope at the same time. The 3′ untranslated region and intergenic sequence of *HDA6* were inserted at the 3′ end of the sequence encoding the EGFP epitope. For epidermis-specific and hair cell-specific expression constructs, a 2,500-bp fragment upstream of the *WER* start codon (Lee and Schiefelbein, 1999) and a 2,500-bp fragment upstream of the *GL3* start codon (Zhang et al., 2003) were separately introduced into pEGAD-EGFP vector to replace the *CaMV35S* promoter. Then, a genomic *HDA6* fragment (from the start codon to the stop codon) was introduced into the above-modified pEGAD-EGFP vector and fused in frame to the 5′ end of the sequence encoding the EGFP epitope to generate WER_{pro}:*HDA6-EGFP*. For $35S_{pro}$:*ETC1*, the coding sequence of *ETC1* was cloned into the pDR vector containing the *CaMV35S* promoter. All the resultant constructs were verified by sequencing. Plasmid DNA was transformed into *Agrobacterium tumefaciens* strain GV3101. Plant transformation was undertaken according to an *A. tumefaciens*-mediated floral dip transformation protocol (Clough and Bent, 1998).

RNA Isolation and qRT-PCR

Total RNA was extracted from root tips of 7-d-old seedlings using the RNeasy Plant Mini kit (Qiagen) according to the manufacturer's instructions. After treatment with DNase I (Promega), complementary DNA was synthesized from 2 μg of total RNA using the SuperScript III Reverse Transcriptase kit (Invitrogen). The qRT-PCR was performed with the Applied Biosystems 7500 Real-Time PCR System using TaKaRa SYBR Premix Ex Taq (Tli RNaseH Plus). Gene-specific primers were used for qRT-PCR (Supplemental Table S1). Relative gene expression was calculated by the ΔΔCT method (Livak and Schmittgen, 2001) using *GAPDH* as an internal control.

The primers used for RNA level detection are listed in Supplemental Table S1.

ChIP Assays

ChIP for acetylated histones H3 and H4 was carried out as described previously (Saleh et al., 2008). Root tips were cut from 7-d-old seedlings and treated with formaldehyde. Extracted chromatin was sheared to an average length of 500 bp by sonication and immunoprecipitated with specific antibodies including anti-acetylated histone H3K9K14 (catalog no. 06-599; Millipore) and anti-acetylated histone H4K5K8K12K16 (catalog no. 06-866; Millipore). The ChIP DNA was analyzed by quantitative PCR.

ChIP for HDA6 protein was carried out with some changes according to Liu et al. (2013b). Seven-day-old seedlings of the $35S_{pro}$:*HDA6-myc* transgenic line (Col background) or Col were harvested and kept in liquid nitrogen. After the samples were ground into powder in liquid nitrogen, the slurry was resuspended with 1× phosphate buffer containing 100 mM Suc, 10 mM dimethyl adipimidate (Sigma; Kurdistani and Grunstein, 2003), and 1% (v/v) formaldehyde. After incubation for 20 min at 4°C, the cross-linking was stopped by incubation with 150 mM Gly for 5 min at 4°C. Chromatin was then extracted and sonicated to produce DNA fragments of about 500 bp. Rabbit antibody anti-c-myc (C3956; Sigma) was used for immunoprecipitation. The ChIP DNA was analyzed by quantitative PCR with Col as a negative control. The commercial myc antibody (Sigma) was confirmed by ChIP-western before use (Supplemental Fig. S7).

The primers used for ChIP assays are listed in Supplemental Table S2.

Microscopy

Transverse serial sections from Arabidopsis root tips were stained with Toluidine Blue to analyze the epidermal cellular pattern (Galway et al., 1994; Xu et al., 2005). For all GUS reporter lines, histochemical analysis was performed as described previously (Masucci et al., 1996). Photographs were taken with a microscope.

Observation of ectopic nonhairs in the mature zone of root was performed as described previously (Caro et al., 2007). Root hair number per millimeter was measured as described previously (Galway et al., 1994).

All EGFP or GFP expression lines were counterstained with 20 μg mL^{-1} PI in water for 1 to 2 min and examined with a Zeiss LSM 710 NLO & DuoScan System. GFP and PI signals were observed sequentially on separate channels, with excitation at 488 nm and detection between bandwidths 493 and 542 nm for GFP and with excitation at 561 nm and detection between bandwidths 566 and 628 nm for PI. For quantitative analysis of root epidermis cellular patterning using reporter-expressing cells, at least 10 roots of 7-d-old seedlings were examined for each line (Wieckowski and Schiefelbein, 2012).

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL data libraries under the following accession numbers: *CPC* (At2g46410), *EGL3* (At1g63650), *GL2* (At1g79840), *GL3* (At5g41315), *WER* (At5g14750), *TTG* (At5g24520), *TRY* (At5g53200), *ETC1* (At1g01380), *SCM* (At1g11130), *GEM* (At2g22475), *HDA6* (At5g63110), and *HDA18* (At5g61070).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. The regulation network of cell fate specification in the Arabidopsis root epidermis, with minor modifications from Schiefelbein et al. (2014).

Supplemental Figure S2. Detection of *HDA6* expression in the root tip of *hda6* mutants and the overexpression line.

Supplemental Figure S3. Complementation of the *hda6* mutant with genomic *HDA6*.

Supplemental Figure S4. *HDA6* expression information retrieved from AREX LITE: The Arabidopsis Gene Expression Database (http://www.arexdb.org/).

Supplemental Figure S5. Schematic representation of the WER_{pro}:*HDA6-EGFP* construct.

Supplemental Figure S6. Detection of expression levels of pattern genes in root tips of CS1895.

Supplemental Figure S7. ChIP-western with myc antibody using total protein extract from Arabidopsis seedlings.

Supplemental Figure S8. Analysis of *ETC1* overexpression lines.

Supplemental Table S1. List of primers used for RNA level detection.

Supplemental Table S2. List of primers used for ChIP assays.

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