

The bromodomain protein GTE6 controls leaf development in *Arabidopsis* by histone acetylation at *ASYMMETRIC LEAVES1*

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The transition from the juvenile to the mature phase during vegetative development in plants is characterized by changes in leaf shape. We show that *GENERAL TRANSCRIPTION FACTOR GROUP E6* (*GTE6*) regulates differences in leaf patterning between juvenile and mature leaves in *Arabidopsis*. *GTE6* encodes a novel small bromodomain-containing protein unique to plants. Mutations in *GTE6* disrupt the formation of elliptical leaf laminae in mature leaves, whereas overexpression of *GTE6* resulted in elongated juvenile leaves. *GTE6* positively regulates the expression of *ASYMMETRIC LEAVES1* (*AS1*), which encodes a myb-domain protein that controls proximodistal patterning of leaves. Using chromatin immunoprecipitation (ChIP) assays, we show that *GTE6* is associated with the promoter and the start of the transcribed region of *AS1* and up-regulates expression of *AS1* through acetylation of histones H3 and H4. Genetic studies demonstrated that *AS1* is epistatic to *GTE6*, indicating that *GTE6* regulates *AS1* during leaf morphogenesis. Chromatin remodeling at *AS1* is a key regulatory mechanism in leaf development, which ensures the continual production of mature leaves following juvenile–adult transition, thereby maintaining the identity of the mature vegetative phase.

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Plant development follows a coordinated and progressive transition from the reproductively incompetent juvenile phase to the mature phase, which is competent for flowering (for review, see Kerstetter and Poethig 1998). The transition from juvenile to mature development involves various physiological and morphological changes, including alterations in leaf structure. Variation in leaf morphology between vegetative phases, also known as heteroblasty, is observed in many plant species, including the model plant *Arabidopsis* (Lawson and Poethig 1995; Telfer et al. 1997). In *Arabidopsis*, juvenile leaves are round with a sharp boundary between the lamina and petiole, whereas the lamina of mature leaves is larger and elliptical, with an obtuse angle between the lamina and petiole (Lawson and Poethig 1995; Theodoris et al. 2003). Heteroblasty is an important developmental pro-

cess, whereby leaf morphology is modified for efficient light capture, because of differences in the light environment and the size of the shoot between juvenile and mature vegetative phases (Day et al. 1997; Kerstetter and Poethig 1998).

Recent studies have indicated that leaf morphogenesis is regulated by post-translational modifications of histones and chromatin remodeling (Wagner 2003). Histones are modified by acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation, which constitutes a code for various regulatory proteins to carry out specific functions, such as transcriptional activation or repression (Strahl and Allis 2000). In *Arabidopsis*, mutations in genes involved in epigenetic silencing, such as *PKL*, which encodes the plant homolog of the mammalian *Mi2* (Eshed et al. 1999), and the polycomb-group genes *CLF* (Goodrich et al. 1997), *EMF2* (Chen et al. 1997), and *FIE* (Katz et al. 2004), resulted in aberrant leaf structures. In addition, mutations in genes involved in heterochromatin formation, such as *LHP1* (Gaudin et al. 2001), *TFL2* (Larson et al. 1998), and *HISTONE H1* (Wierzbicki and Jerzmanowski 2004), also lead to changes in leaf shape and size. In transgenic *Arabidopsis* containing an antisense construct of the histone deacetylase gene *ATHD1*, which

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resulted in increased histone acetylation, leaf shape was dramatically altered, and juvenile leaves resembled mature leaves (Tian and Chen 2001). This suggests that the expression of genes involved in leaf-axis specification is regulated by acetylation of histones and chromatin remodeling (Tian and Chen 2001). Acetylated histone tails are recognized by the bromodomain, which is a conserved protein module of ~110 amino acid residues that binds acetylated lysine residues (Dhalluin et al. 1999; Ladurner et al. 2003). The bromodomain module is present in various basal transcription factors and coactivators, and links acetylated histones and transcriptional activation (Dhalluin et al. 1999; Ladurner et al. 2003).

Plants possess an open growth system and are constantly forming new vegetative organs (Poethig 1990; Telfer et al. 1997). Leaf primordia that are initiated from the shoot apical meristem have to be specified to develop into a juvenile leaf or a mature leaf, depending on the vegetative phase of the plant. This regulatory process is nonexistent in animals, because maturation involves differentiation of organ primordia that are formed during embryogenesis, along with recruitment of new cells (Thisse and Zon 2002). Here, we show that *GENERAL TRANSCRIPTION FACTOR GROUP E6* (*GTE6*), which encodes a bromodomain-containing protein, plays an essential role in controlling differences in the development of primordia produced during juvenile and mature phases. *GTE6* regulates the development of mature leaf shape through acetylation of histones present on *ASYMMETRIC LEAVES1* (*AS1*), a gene involved in leaf-axis specification in *Arabidopsis* (Byrne et al. 2000, 2001). Our data reveal the functions of a small bromodomain gene unique to plants and indicate a network of transcription factors that regulates leaf patterning during juvenile–adult transition.

Results

High GTE6 expression is correlated with mature leaf shape

GTE6 has eight exons and encodes a protein of 369 amino acid residues, with the bromodomain motif spanning residues 116–194 (Fig. 1A). To identify the transcription start site of *GTE6*, the 5' end of the *GTE6* transcript was examined by 5' rapid amplification of cDNA ends (RACE), followed by PCR. The transcription start site was mapped to 73 bp upstream of the translation start site (Fig. 1A).

In wild-type *Arabidopsis*, *GTE6* transcripts are most abundant in flowers; low in roots, leaves, and siliques; and undetectable in 5-d-old seedlings (Fig. 1B). In the basal rosette leaves of 21-d-old wild-type plants, *GTE6* transcripts are more abundant in leaves 6 and 7, which possess narrow elliptical laminae, than in leaves 1–4, which have round laminae (Fig. 1C,D), suggesting a possible correlation between *GTE6* expression and the formation of elliptical leaf laminae in mature leaves.

Differences in *GTE6* expression between the juvenile and mature leaves in 21-d-old plants are not likely to be

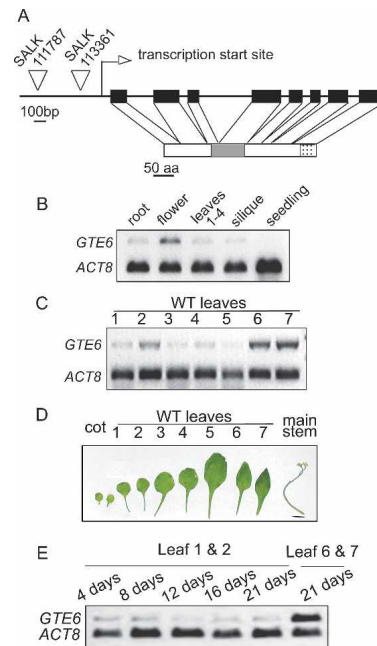


Figure 1. *GTE6* and its expression in *Arabidopsis*. (A) Schematic diagram of *GTE6*. Solid rectangles indicate exons. Arrowheads mark the sites of T-DNA insertions. The open rectangle represents amino acid residues 1–369 of *GTE6*, showing the positions of the bromodomain (shaded box) and the nuclear targeting signal (dotted box). (B) Transcript abundance of *GTE6*, as analyzed by RT-PCR using *ACTIN8* (*ACT8*) as an internal loading control, in various tissues of 21-d-old wild-type Columbia plants. Seedlings were 5 d old. (C) RT-PCR analyses of *GTE6* transcripts in individual leaves of the basal rosette of 21-d-old wild-type Columbia plants. Leaves are numbered in order of appearance, with leaf 7 being the youngest leaf. (D) Individual leaves of the basal rosette of 21-d-old wild-type Columbia. Bar, 1 cm. (E) RT-PCR analyses of *GTE6* transcripts in leaves at various times during leaf elongation and maturation. Leaves 1 and 2 were collected 4, 8, 12, 16, or 21 d after appearance.

due to differences in leaf age, because *GTE6* transcript levels are similar in leaves 1 and 2 at 4–21 d after leaf initiation, suggesting that *GTE6* expression does not change during various times of leaf elongation and maturation (Fig. 1E).

Mutations in GTE6 result in alteration of leaf shape

To investigate whether *GTE6* may control the development of leaf shape, we analyzed mutants from the SALK collection (Alonso et al. 2003) that contained transferred DNA (T-DNA) insertions in the promoter region of the *GTE6* gene (Fig. 1A). The effect of the mutations is most obvious in leaves 6 and 7, which have round laminae (Fig. 2A; width/length ratios in Supplementary Fig. S1), rather than the elliptical laminae of leaves 6 and 7 of the wild-type plants (Fig. 1D). The T-DNA insertions resulted in fewer *GTE6* transcripts in leaf 7 (Fig. 2B), suggesting misregulation of *GTE6* expression. The transcript abundance of *GTE6* in leaf 7 of the insertion mu-

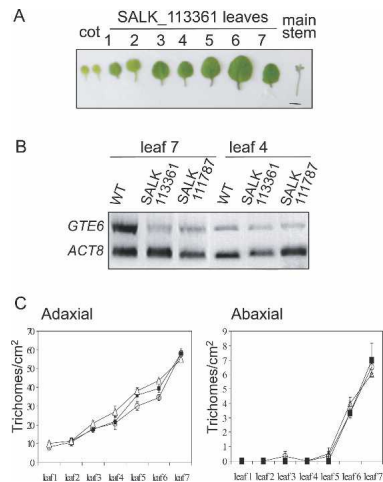


Figure 2. T-DNA insertion mutants of *GTE6*. (A) Basal rosette leaves of 21-d-old insertion mutant SALK_113361. Bar, 1 cm. (B) RT-PCR analysis of *GTE6* transcripts in leaves 7 and 4 of 21-d-old Columbia wild-type and T-DNA insertion mutants. (C) Trichome numbers on the adaxial (left) and abaxial (right) surfaces of wild-type Columbia (squares), SALK_13361 (triangles), and SALK_111787 (circles). The plants were germinated on MS agar, and trichomes were counted 4 wk later. The plants flowered after producing seven leaves. Error bars indicate standard error of the mean.

tants was similar to leaf 4 of the wild-type and the insertion mutant plants, suggesting that the T-DNA insertions have disrupted the activation of *GTE6* expression in the mature leaves (Fig. 2B). Thus, elevated expression of *GTE6* in leaves 6 and 7 of wild-type plants is important for the development of the elliptical leaf lamina.

Phase transition during vegetative development is also characterized by changes in trichome distribution (Telfer et al. 1997). In *Arabidopsis*, juvenile leaves (leaves 1 to 4) have trichomes present only on the adaxial (upper) surface, whereas mature leaves (leaves 5 and above) possess trichomes on both adaxial and abaxial (lower) surfaces (Telfer et al. 1997). Trichome distribution on the rosette leaves of the insertion mutants was similar to that of the wild-type plants (Fig. 2C), indicating that *GTE6* does not regulate phase transition per se but is involved in controlling development of mature leaf shapes.

To investigate further the regulation of leaf development by *GTE6*, we transformed wild-type plants with a 35S::*GTE6* construct containing the cauliflower mosaic virus (CaMV) 35S promoter fused to the *GTE6* cDNA, resulting in more *GTE6* transcripts (Fig. 3A). Elevated *GTE6* expression resulted in juvenile leaves showing an elongated and elliptical shape (Fig. 3B; width/length ratios in Supplementary Fig. S1). We also analyzed mutants possessing decreased amounts of *GTE6* transcripts, generated using a 35S::*RNAiGTE6* construct containing the CaMV 35S promoter linked to two copies of a 524-bp region of the *GTE6* cDNA in inverted orientations. Database analysis of this 524-bp sequence did not identify any other *Arabidopsis* genes containing similar nucleotide

sequences of 15 bp or more, suggesting that the 35S::*RNAiGTE6* construct was specific for *GTE6*. Decreased *GTE6* transcript levels in the 35S::*RNAiGTE6* plants (Fig. 3C) resulted in shorter and broader leaf laminae in mature leaves, as compared with the corresponding leaves in the wild-type plants (Fig. 3D; width/length ratios in Supplementary Fig. S1). Control plants transformed with empty vectors were morphologically similar to wild-type plants, indicating that the phenotype of the leaves of plants containing the 35S::*GTE6* and 35S::*RNAiGTE6* constructs was not the result of transformation procedures (data not shown). Altered amounts of *GTE6* transcripts in the 35S::*GTE6* and 35S::*RNAiGTE6* plants did not alter the rate of leaf production, the flowering time, or the total number of leaves in the basal rosette, suggesting that the vegetative phase was not affected. These data indicate that *GTE6* is involved in the development of the leaf lamina in mature leaves. No change in flower morphology, silique structure, or root length was observed in the mutants.

GTE6 positively regulates the expression of *AS1*

The proximodistal axis of the leaf is established in part by *AS1* in *Arabidopsis* (Byrne et al. 2000, 2001). Loss-of-

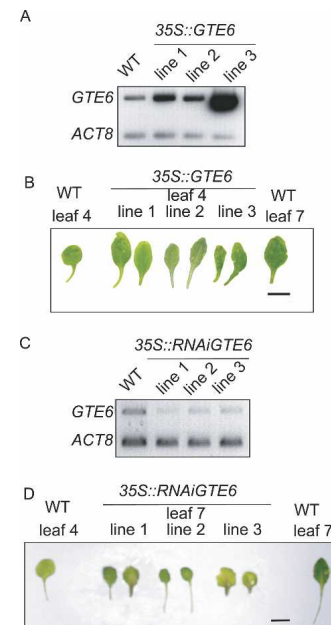


Figure 3. Altered expression of *GTE6*. (A) Transcript abundance of *GTE6* in shoots of three independently transformed lines of 35S::*GTE6* plants, analyzed by quantitative RT-PCR using *ACT8* as an internal control. (B) Leaves of three individually transformed lines of 35S::*GTE6* plants. Bar, 1 cm. (C) Transcript abundance of *GTE6* in shoots of three independently transformed lines of 35S::*RNAiGTE6* plants, transformed with an RNAi construct containing two copies of *GTE6* cDNA sequences in reverse orientations, separated by a 1-kbp intron of the pea *Lip1* gene. This construct should produce a double-stranded RNA upon transcription, which should target the RNA degradation machinery to *GTE6* transcripts to induce silencing. (D) Leaves of three individually transformed lines of 35S::*RNAiGTE6* plants. Bar, 1 cm.

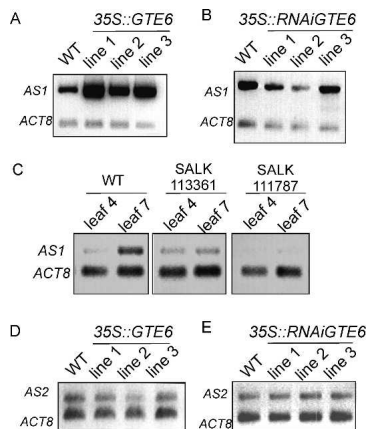


Figure 4. Expression of *AS1* and *AS2* in *GTE6* mutants. (A) Expression of *AS1* in shoots of 21-d-old *35S::GTE6* plants, analyzed by RT-PCR. (B) RT-PCR analyses of *AS1* in shoots of 21-d-old *35S::RNAiGTE6* plants. (C) RT-PCR analyses of *AS1* transcripts in leaves 4 and 7 of wild-type Columbia and T-DNA insertion mutants. (D) Expression of *AS2* in shoots of 21-d-old *35S::GTE6* plants. (E) RT-PCR analyses of *AS2* in shoots of 21-d-old *35S::RNAiGTE6* plants.

function mutations in *AS1* led to shorter, broader, and occasionally lobed laminae, whereas overexpression resulted in elongated and elliptical juvenile leaves (Byrne et al. 2000; Theodoris et al. 2003). Because the phenotypes of the *GTE6* mutants are similar to the *AS1* mutants and *GTE6* is likely to regulate leaf shape by regulating the expression of genes involved in leaf development, we investigated *AS1* transcript levels in the *GTE6* mutants. Elevated *GTE6* expression in the *35S::GTE6* plants resulted in more *AS1* transcripts (Fig. 4A), whereas decreased expression of *GTE6* in the RNAi plants resulted in fewer *AS1* transcripts (Fig. 4B). In wild-type plants, *AS1* transcripts in leaf 7 were more abundant than in leaf 4 (Fig. 4C), consistent with the observation that *GTE6* transcripts were more abundant in leaf 7 than leaf 4 (Fig. 1C). No difference in the amount of *AS1* transcripts was observed between leaves 7 and 4 of the T-DNA insertion mutants, suggesting misregulation of *AS1* expression in the insertion mutants (Fig. 4C). We also examined the expression of *AS2*, which genetically interacts with *AS1* to regulate leaf patterning in *Arabidopsis* (Semiarti et al. 2001). The expression of *AS2* was not altered in the *GTE6* overexpressing mutants (Fig. 4D) or in *GTE6* RNAi plants (Fig. 4E). The expression of other genes involved in leaf development, such as *AN* (Tsuge et al. 1996), *CLF* (Goodrich et al. 1997), *DRL* (Nellissen et al. 2003), *ROT3* (G.-T. Kim et al. 1999), and *TPC2* (Palatnik et al. 2003) was also investigated. However, no change in the transcript levels of these genes was observed in the *GTE6* mutants, suggesting that *GTE6* is not regulating the expression of these genes (Supplementary Fig. S2).

Although *as1* and *GTE6* T-DNA insertion mutants have similar altered mature leaf phenotypes, they are different in other aspects of leaf development; e.g., *GTE6* T-DNA insertion mutants have flat leaf laminae

whereas *as1* has curly leaf blades (Fig. 5A,B). Double mutants of *as1* and *GTE6* T-DNA insertion mutant have curly leaves and are morphologically more similar to *as1* than *GTE6* mutants, suggesting that *AS1* is epistatic to *GTE6* (Fig. 5C).

When *GTE6* was overexpressed in *as1*, no change in leaf shape was observed in any of the overexpressing plants, further suggesting that *GTE6* regulates leaf development through *AS1* (Fig. 5D,E). Wild-type plants overexpressing *AS1* have a phenotype similar to *35S::GTE6* plants; i.e., elliptical leaves 3 and 4, implicating both genes in control of mature leaf shape (Fig. 5F,G). These results provide genetic evidence that *AS1* acts downstream of *GTE6*.

GTE6 associates with the promoter and intron of *AS1*

The location of the *GTE6* protein in the cell was examined using *Arabidopsis* plants stably transformed with a *35S::GTE6-GFP* construct, which expresses a *GTE6*-green fluorescent protein (GFP) fusion protein. The *GTE6-GFP* fusion protein is localized to the nucleus (Fig. 6A), which is consistent with its predicted role as a transcription factor. A nuclear-targeting signal is predicted in the C-terminal region of *GTE6* (Fig. 1A).

To investigate whether the expression of *AS1* is directly regulated by *GTE6*, we examined the association

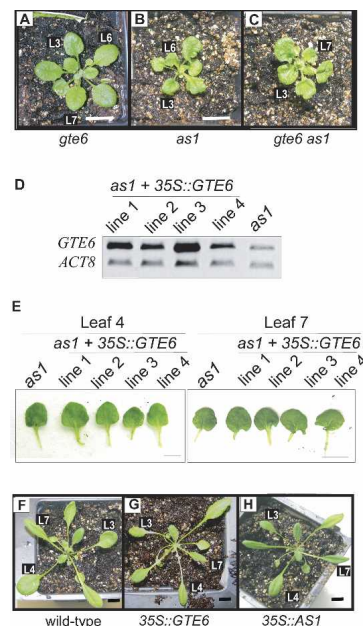


Figure 5. Genetic interactions between *AS1* and *GTE6*. (A) *GTE6* T-DNA insertion mutant SALK_113361. (B) *as1* mutant. (C) Double mutant of *as1* and *GTE6* T-DNA insertion mutant (SALK_113361). (D) RT-PCR analysis of transcript abundance of *GTE6* in four lines of *as1* transformed with a *35S::GTE6* construct. (E) Leaves of the four lines of *as1* overexpressing *GTE6*. Bar, 1 cm. (F) Wild-type Columbia. (G) Columbia containing *35S::GTE6*. (H) Columbia containing *35S::AS1*. Bar, 1 cm. (L) Leaf.

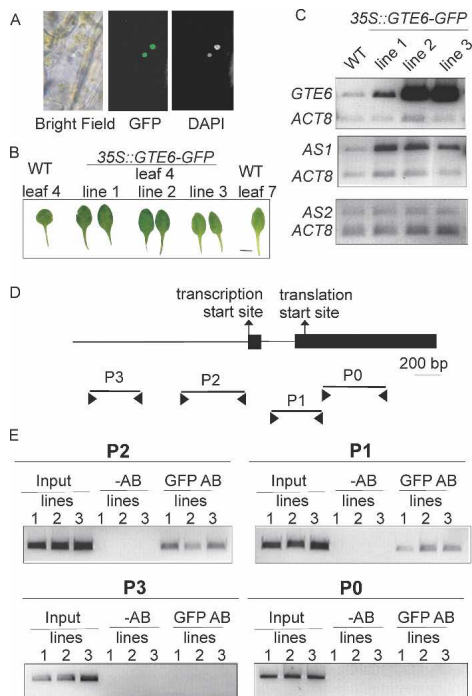


Figure 6. Association of GFP-tagged GTE6 with the *AS1* gene in *35S::GTE6-GFP* plants. (A) Confocal images of hypocotyl cells in a seedling of a *35S::GTE6-GFP* plant (line 2), showing GFP fluorescence and DAPI staining of DNA. The GTE6-GFP fusion in the other lines of *35S::GTE6-GFP* plants were similarly localized to the nucleus. (B) Leaf 4 of two plants from each of the three independently transformed lines of *35S::GTE6-GFP* plants. Bar, 1 cm. (C) Analysis of *GTE6*, *AS1*, and *AS2* transcripts in shoots of 21-d-old plants expressing the *GTE6-GFP* fusion by quantitative RT-PCR. *ACT8* was used as an internal loading control. (D) Location of primers designed to amplify the upstream region (P3), the promoter (P2), the 3' end of the intron and 5' end of exon 2 (P1), and exon 2 (P0) of *AS1* for ChIP assays. Exons are indicated by solid rectangles. (E) ChIP was performed using three independently transformed lines of 21-d-old *35S::GTE6-GFP* plants. (Input) PCR on DNA present in the chromatin extract used for immunoprecipitation; (-AB) ChIP with no antibody; (GFP AB) ChIP with antibodies to GFP. Similar results were obtained in triplicates of ChIP reactions performed on each of the three lines.

of GTE6 with various regions of *AS1* with chromatin immunoprecipitation (ChIP) experiments, using *Arabidopsis* plants expressing a GTE6-GFP fusion protein. Linking GFP to the C terminus of GTE6 did not abolish the function of the protein, as shown by the phenotypes of wild-type plants transformed with *35S::GTE6-GFP*, which were similar to wild-type plants transformed with *35S::GTE6*; i.e., elongated and elliptical laminae in leaves 1–4. Figure 6B shows leaf 4 of three independently transformed lines of *35S::GTE6-GFP* plants. Furthermore, *AS1* transcripts were increased in the *35S::GTE6-GFP* plants (Fig. 6C), as well as in the *35S::GTE6* plants (Fig. 4A), indicating that both GTE6-GFP and GTE6 up-regulated the expression of *AS1*. *AS2* transcripts remained unchanged in the *35S::GTE6-GFP* plants (Fig. 6C), as in the *35S::GTE6* plants (Fig. 4D).

ChIP was performed using antibodies to GFP, as described previously (Chua et al. 2001; Gendrel et al. 2001). Coprecipitated DNA was analyzed by PCR using primer pairs that amplify various regions of *AS1* (Fig. 6D). In three independently transformed lines of *35S::GTE6-GFP* plants, the GFP antibodies coprecipitated the promoter (region P2), and the 3' end of the intron and the 5' end of exon 2 (region P1) of *AS1* (Fig. 6E). The GFP antibodies did not coprecipitate regions P3 and P0 of *AS1*; these sequences were undetected even after forty rounds of PCR. GTE6 thus regulates expression by associating with a 1-kbp region containing the promoter and the start of the transcribed region of *AS1*, which are likely to be important regulatory regions for transcriptional control (Fig. 6E).

GTE6 activates expression by mediating acetylation of histones

We next examined histone acetylation states at *AS1* to investigate whether *GTE6* may regulate expression through acetylation of histones. ChIP was performed using leaves 1–4 of wild-type Columbia and *35S::GTE6* plants. Histones H3 associated with the promoter and the transcribed region of *AS1* (regions P2, P1, and P0) were more acetylated in leaves 1–4 of the *35S::GTE6* plants, compared with the wild-type plants (Fig. 7A–C).

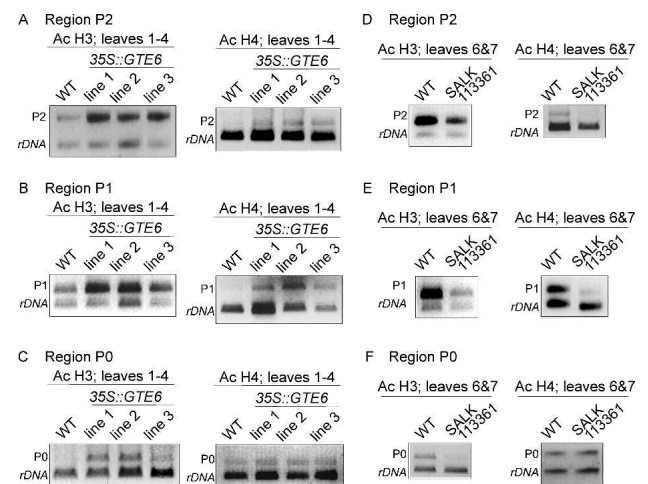


Figure 7. Histone acetylation at *AS1* in *GTE6* mutants. ChIP assays were performed using antibodies to acetylated N-terminal tails of histones H3 (Ac H3), or H4 (Ac H4). (A–C) Histone acetylation at P2, P1, and P0 of *AS1* was investigated in leaves 1–4 of low-expressing (line 2), medium-expressing (line 1), and high-expressing (line 3) lines of 21-d-old *35S::GTE6* plants. The positions of primers used to amplify regions P2, P1, and P0 of *AS1* are indicated in Figure 6D. *18S rDNA* was used as an internal control. Similar results were obtained in triplicates of ChIP reactions performed on each of the three lines. (D–F) Effect of misregulation of *GTE6* expression on histone acetylation status of regions P2, P1, and P0 of *AS1* in the insertion mutant SALK_113361. ChIP assays were performed using leaves 6 and 7 of 21-d-old plants. Similar results were obtained in three independent replicates of ChIP.

Histone H4 acetylation was also increased at regions P2 and P1 of *AS1* in the *35S::GTE6* plants (Fig. 7A,B). Histone H4 acetylation at region P0 of exon 2 was unaltered (Fig. 7C). Elevated *GTE6* expression in the *35S::GTE6* plants thus resulted in increased histone H3 and H4 acetylation at the promoter and the start of the transcribed region of *AS1*, regions where the GTE6–GFP fusion protein binds (Fig. 6E). No change in histone H3 and H4 acetylation at the upstream region P3 (–1855 to –1367, with +1 as translation start site) was observed (data not shown).

Histone acetylation at *AS1* was examined in leaves 6 and 7 of a T-DNA insertion mutant to determine whether decreased *GTE6* expression resulted in decreased histone acetylation. Leaves 6 and 7 were investigated because the shapes of these leaves were most different between the insertion mutant and the wild-type plants (Figs. 1D, 2A). The promoter and the transcribed regions of *AS1* (regions P2, P1, and P0) in leaves 6 and 7 of wild-type plants were associated with high histone H3 and H4 acetylation, compared with leaves 1–4 of wild-type (Fig. 7A–F), again correlating increased histone acetylation with mature leaf shape. In leaves 6 and 7 of the insertion mutant, histone H3 acetylation at the promoter and transcribed region of *AS1* (regions P2, P1, and P0) was lower than in the wild-type plants (Fig. 7D–F). Histone H4 acetylation at regions P2 and P1 of *AS1* was also decreased in the insertion mutant (Fig. 7D,E). Thus, lower *GTE6* expression in leaves 6 and 7 of the T-DNA insertion mutant is associated with decreased acetylation of histones H3 and H4 at *AS1*, as compared with wild-type plants. No difference in histone H4 acetylation at region P0 of exon 2 was observed between the insertion mutant and the wild-type plants (Fig. 7F).

GTE6 encodes a nuclear protein unique to plants

Following the complete sequencing of the *Arabidopsis* genome, *GTE6* was annotated as a BET (bromodomain–Extraterminal) gene, because it contains a bromodomain and an Extraterminal (ET) domain, and was classified as a member of the *GTE* family of transcription factors (Pandey et al. 2002). The function of the ET domain is unknown but has been proposed to be involved in protein–protein interactions (Lygerou et al. 1994; Jeanmougin et al. 1997). The BET class of proteins includes the yeast BDF1 (Lygerou et al. 1994), the *Drosophila* FSH (Chua and Roeder 1995), and the vertebrate Brd2/Ring3/fsrg2, Brd4/HUNKI/MCAP, and Brd5/BRDT (Houzelstein et al. 2002). However, *GTE6* was not among the 11 putative BET proteins proposed for *Arabidopsis* (Florence and Faller 2001). The C terminus of *GTE6* contains only 9 of the 30 residues conserved in the ET domain and lacks the serine-rich motif, known as the SEED motif, that is characteristic of BET proteins (Supplementary Fig. S3; Florence and Faller 2001). The sequences flanking the bromodomain of *GTE6* also do not possess similarity to animal and yeast BET proteins. Furthermore, animal and yeast BET proteins contain 2 bromodomains, whereas *GTE6* contains a single bromodomain. Thus,

GTE6 is not likely to be a plant homolog of the animal BET proteins. Database searches indicate that genes that possess high sequence similarities with *GTE6* are present in pea, maize, and rice but not in unicellular organisms and animals, suggesting that this class of genes is unique to plants.

Discussion

GTE6 up-regulates expression of *AS1* during leaf development

Our findings indicate that *AS1* is a target gene of *GTE6*, and its expression is regulated by *GTE6* through acetylation of histones. In wild-type *Arabidopsis*, high *GTE6* expression in mature leaves, as compared with juvenile leaves, results in increased histone H3 and H4 acetylation at the promoter and transcribed region of *AS1*, leading to activation of *AS1* expression. This leads to lamina elongation along the proximodistal axis and contributes to the elliptical shape in mature leaves.

GTE6 associates with the promoter and the first exon and intron of *AS1* in a 1-kbp region, and increases the acetylation states of histones H3 and H4 present on this region. Increased histone H3 acetylation was also observed at exon 2, which is not associated with *GTE6*, in overexpressing mutants. *GTE6* may increase histone acetylation at *AS1* by binding to acetylated histones and protecting them from the enzymatic actions of histone deacetylases. This has been observed in yeast, where the BET protein Bdf1p shields acetylated histone H4 from deacetylation by Sir2p, which is a histone deacetylase (Ladurner et al. 2003). Alternatively, *GTE6* may function as an adaptor protein that associates with histone acetyltransferases to increase acetylation levels (Hassan et al. 2001). An investigation of the proteins that interact with *GTE6* in vivo may identify the mechanism of *GTE6*-mediated histone acetylation.

The mature leaves of *as1* and *gte6* homozygous plants display round laminae, suggesting that both genes are involved in the specification of adult leaf phenotype. The observation that the *as1 gte6* double mutant is more similar to *as1* than *gte6* indicates that *as1* is epistatic to *gte6* in the development of mature leaves. We showed that *AS1* is indeed a downstream target gene of *GTE6* using ChIP experiments. Although the mature leaves are similar in both the *as1* and *gte6* mutants, there are slight phenotypic differences; *as1* has curly leaf laminae, and *gte6* has flat leaves (Fig. 5A,B). This is likely to be due to differences in *AS1* expression; *as1* is a null mutant and does not contain any functional *AS1* protein (Byrne et al. 2000), whereas *gte6* expresses *AS1*, albeit at a lower level than wild-type plants (Fig. 4C). Thus, the phenotypes of *as1*, such as broader and curly leaf lamina and shorter petioles, are stronger than those of *gte6*.

Overexpression of *GTE6* in *Arabidopsis* resulted in elongated juvenile leaves, whereas RNAi depletion resulted in round mature leaves. However, overexpression of *GTE6* in an *as1* background did not alter leaf shape, further supporting the notion that *GTE6* and *AS1* are in

the same genetic pathway, and that *GTE6* requires a functional *AS1* gene to regulate leaf morphogenesis. *AS1* is a myb-domain protein and functions as a transcription factor (Byrne et al. 2000). *AS1* and its homologs *ROUGH SHEATH2* in maize and *PHANTASTICA* in *Antirrhinum*, play central roles in leaf patterning (Byrne et al. 2000, 2002). *AS1* may modulate leaf shape by affecting the direction of cell division and/or elongation. In wild-type leaves, epidermal cell files run almost parallel to the petiole, indicating that cells elongate in one general direction to give an elliptical leaf lamina (Meijer and Murray 2001). However, in *as1* mutants, files of elongated cells are arranged perpendicular to the petiole, resulting in round leaf laminae (Byrne et al. 2000). *AS1* forms a complex with *AS2* to repress the transcription of the *KNOX* genes *KNAT1* and *KNAT2*, which encode homeobox transcription factors in the apical meristem (Byrne et al. 2002; Theodoris et al. 2003). Consistently, the leaf phenotypes of *gte6* mutants are similar to *as2* mutants (data not shown), suggesting that the downstream target genes of *GTE6* and *AS2* are common. However, because *GTE6* does not regulate the expression of *AS2* (Fig. 4D,E), the genes downstream of *AS2* are likely to be controlled by *GTE6* through *AS1*.

GTE6 specifies mature leaf development

Leaf morphogenesis is regulated by various chromatin remodeling genes. For example, mutations in *PKL* (Eshed et al. 1999), which is similar to the mammalian repressor Mi2, and *CLF* (Goodrich et al. 1997), *EMF2* (Chen et al. 1997), and *FIE* (Katz et al. 2004), which encode polycomb-group proteins, result in aberrant leaf structures. In addition, abnormalities in leaf development were observed in mutants of *LHP1* (Gaudin et al. 2001), *TFL2* (Larsson et al. 1998), and *HISTONE H1* (Wierzbicki and Jerzmanowski 2004), which encode proteins involved in heterochromatin formation. These genes repress transcription through histone deacetylation and DNA methylation and regulate leaf morphogenesis by silencing flowering and embryonic genes in leaves (Goodrich et al. 1997; Eshed et al. 1999). Our data show that acetylation of histones regulates a very different aspect of leaf morphogenesis; i.e., specification of differences between juvenile and mature leaf patterning. Interestingly, antisense inhibition of the histone deacetylase gene *ATHD1* in *Arabidopsis* resulted in increased amounts of acetylated histones H3 and H4, and also elongated and serrated juvenile leaves that resembled mature leaves, further correlating mature leaf development with acetylation of histones (Tian and Chen 2001). Chromatin remodeling thus plays a central role in regulation of leaf development, where mechanisms of gene silencing and heterochromatin formation regulate tissue-specific gene expression, and histone acetylation controls leaf patterning.

Plants possess an open growth system and are constantly forming new leaf primordia at the meristems (Poethig 1990; Byrne et al. 2000). During transition into the mature vegetative phase, mature leaves are not de-

rived from juvenile leaves but develop from newly formed leaf primordia. A mature plant thus possesses both juvenile and mature vegetative organs, with the older juvenile leaves present at the base of the plant and the younger mature leaves at the apices (Poethig 1990; Telfer et al. 1997). Both the juvenile and the mature vegetative developmental phases are maintained in the plant, because shoots initiated from the juvenile parts possess juvenile traits, whereas shoots arising from the mature parts produce mature leaves (Telfer et al. 1997). Moreover, it is extremely difficult to convert the mature phase back to the juvenile phase (Poethig 1990). It has long been proposed that the mechanism that maintains developmental phases in plants is epigenetic (Poethig 1990), and phase transition is associated with changes in genomic DNA methylation (Fraga et al. 2002; Baurens et al. 2004), although the regulatory process involved has not been elucidated. Our data indicate that chromatin remodeling at *AS1* is necessary for the adult leaf phenotype. This epigenetic control of mature leaf shape by acetylation of histones would provide plants with the mechanism to continue producing mature leaves and not to revert into initiating juvenile leaves, following transition into the mature vegetative phase.

Heteroblasty reflects plasticity in plant development, where leaf morphology is modified during the mature vegetative phase for efficient light capture for photosynthesis (Day et al. 1997). In *Arabidopsis*, the elliptical mature leaf possesses almost twice as much lamina as a round juvenile leaf of the same width. Thus, the adult leaf shape minimizes overlap between leaves while increasing lamina area available for light capture. Heteroblasty is associated with aging, which occurs after the juvenile vegetative phase (Kerstetter and Poethig 1998). Interestingly, treatment with histone deacetylase inhibitors increases life span and retards aging in *Drosophila* (Chang and Min 2002; Kang et al. 2002). In addition, overexpression of the histone deacetylase gene *Sir2* in yeast and *Caenorhabditis elegans* (Imai et al. 2000; Tissenbaum and Guarente 2001), and deletion of the histone deacetylase gene *RPD3* in yeast (S. Kim et al. 1999), delay the aging process, suggesting that genes associated with maturation and aging are regulated by deacetylation of histones. We show that elongated elliptical leaf lamina, an adult trait in *Arabidopsis*, is controlled by acetylation of histones. It is interesting that regulation of gene expression during maturation appears to involve changes in histone acetylation levels in both animals and plants.

In summary, we propose that *GTE6* plays a fundamental role in regulating leaf patterning during juvenile-to-adult transition in plants, and the mechanism involves chromatin remodeling at *AS1* through acetylation of histones. *AS1* in turn represses the transcription of *KNAT1* and *KNAT2*, which encode homeobox proteins. Regulation of leaf shape associated with heteroblasty is thus a highly regulated process, involving a network of transcription factors. *GTE6* and its plant homologs are likely to be plant-specific proteins adapted to maintain adult leaf shapes during the mature vegetative phase through acetylation of histones.

Materials and methods

Plants and growth conditions

Seeds of mutant and wild-type Columbia were surface-sterilized and germinated on 1/2 MS agar (0.24% MS salts, 0.8% agar at pH 5.9). The seeds were treated in the dark for 4 d at 4°C and transferred to 25°C under a 24-h cycle of 16 h PAR (150 $\mu\text{mol photons m}^{-2}\text{/s}$) and 8 h dark. Unless otherwise stated, all experiments were performed using 21-d-old plants grown on 1/2 MS agar in tissue culture plates. The T-DNA insertion mutants SALK_111787 and SALK_113361 were obtained from the SALK Institute (Alonso et al. 2003). Seeds of the *as1* mutant were obtained from the Arabidopsis Biological Resource Centre. Seeds of Columbia containing 35S::*AS1* were provided by Michael Freeling (University of California, Berkeley, CA).

DNA constructs and plant transformation

The transcription start site of *GTE6* was mapped by 5' RACE using the FirstChoice RLM-RACE kit (Ambion, Inc.), using the method of Shaefer (1995). The primers used for the RACE reactions were GTE6raceR4 and GTE6raceR6 (Supplementary Table S1). The PCR products were sequenced to identify the transcription start site. The 35S::*GTE6* construct was generated by inserting the cDNA of *GTE6* into the BamHI site of pROK2 (Baulcombe et al. 1986). The 35S::*RNAiGTE6* construct was generated using the intermediate vector pJH21, which contains intron 4 of the pea *Lip1* gene (Sullivan and Gray 2000) inserted into the SalI–BamHI sites of pBCSK + phagemid (Stratagene). A 524-bp region of *GTE6* (+587 to +1100 of *GTE6* cDNA, with +1 as the translation start site) was inserted into the BglII–SalI sites of pJH21, and the same sequence, but in the reverse orientation, was inserted into the BamHI–SacI sites. The 524-bp region of *GTE6* was obtained by PCR amplification. The DNA fragment containing the two copies of *GTE6* separated by the intron was released by digestion with BglII and SacI, and inserted into pROK2. The 35S::*GTE6–GFP* construct was generated by inserting the cDNA of *GTE6* into an intermediate vector pUCGFP (Haseloff et al. 1997), which contained the CaMV 35S promoter sequence, *GFP*, and the terminator of *nos* in pUC19. The 35S::*GTE6–GFP* sequence was excised by digestion with HindIII and EcoRI, and inserted into pROK2. Wild-type Col or *as1-1* mutant was transformed with 35S::*GTE6*, 35S::*RNAiGTE6*, or 35S::*GTE6–GFP* with *Agrobacterium* strain C58C1 using the floral dip method (Clough and Bent 1998).

Generation of double mutant

The double mutant of *as1-1* and *GTE6* T-DNA insertion mutant (SALK_113361) was generated by pollinating *as1* flowers with pollen from SALK_113361. Seeds collected from F1 plants were germinated, and the segregating F2 population was screened for double mutants by PCR using the primers LBB1, GTE6p-F2, GTE6p-F3, GTE6p-R2, *AS1-f*, and *AS1-r* (Supplementary Table S1).

Confocal microscopy

Epidermal peels from *Arabidopsis* leaves were stained with DAPI (10 mg/mL, Roche) for 15 min, washed with water, placed on glass slides, and examined by confocal laser scanning microscopy (Leica Microsystems) for GFP and DAPI–DNA fluorescence.

RNA analysis

RNA was purified using Tripure reagent (Roche), and reverse transcription was performed as previously described (Chua et al. 2004). Semi-quantitative duplex PCR was performed using primers specific for *ACT8*, *AS1*, *GTE6*, *AN*, *CLF*, *ROT3*, *AS2*, *DRL*, or *TCP2* (Supplementary Table S1). PCR reactions were first performed with various dilutions of the template DNA to ensure that the PCR conditions were within the quantitative range of the amplification reaction. Typically, 28 cycles were used.

ChIP

ChIP experiments were performed as described previously (Chua et al. 2001; Gendrel et al. 2001). Approximately 0.1–0.8 g leaves (leaves 1–4 or 6 and 7, as described in the figure legends) of 21-d-old *Arabidopsis* plants grown on 1/2 MS agar in tissue culture plates were fixed with 1% formaldehyde (Sigma) under vacuum for 15 min. The plant material was ground in liquid nitrogen, and chromatin was extracted. The chromatin was sheared into fragments ranging from 400 bp to 1 kbp by sonication. The sonicated chromatin was immunoprecipitated with 10 μL of anti-acetylated histone H4 (Upstate Biotechnology), 10 μL of anti-acetylated histone H3 (Upstate Biotechnology), or 5 μL of anti-GFP (Abcam). DNA was amplified using primers specific for *18S rDNA*, and various regions of *AS1* (Supplementary Table S1). PCR reactions were first performed with various dilutions of the template DNA to ensure that the PCR conditions were within the quantitative range of the amplification reaction. Coprecipitated DNA was dissolved in 20 μL of TE, and typically 1 μL was used for PCR analyses. For total input samples, DNA was extracted from an aliquot of sonicated chromatin, dissolved in 80 μL of TE, and 1 μL was used for PCR. Twenty-eight cycles were used to amplify regions P1, P2, P3, or P0 of *AS1*, and 20 cycles were used to amplify the *18S rDNA* sequence. Regions of *AS1* were first amplified using *AS1* primers for 8 cycles, followed by the addition of *18S rDNA* primers, and the PCR was performed for another 20 cycles.

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