ASYMMETRIC LEAVES2 and Elongator, a Histone Acetyltransferase Complex, Mediate the Establishment of Polarity in Leaves of Arabidopsis thaliana

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Leaf primordia are generated around the shoot apical meristem. Mutation of the ASYMMETRIC LEAVES2 (AS2) gene of Arabidopsis thaliana results in defects in repression of the meristematic and indeterminate state, establishment of adaxial–abaxial polarity and left–right symmetry in leaves. AS2 represses transcription of meristem-specific class 1 KNOX homeobox genes and of the abaxial-determinant genes ETTIN/ARF3, KANADI2 and YABBY5. To clarify the role of AS2 in the establishment of leaf polarity, we isolated mutations that enhanced the polarity defects associated with as2. We describe here the enhancer-of-asymmetric-leaves-two1 (east1) mutation, which caused the formation of filamentous leaves with abaxialized epidermis on the as2-1 background. Levels of transcripts of class 1 KNOX and abaxial-determinant genes were markedly higher in as2-1 east1-1 mutant plants than in the wild-type and corresponding single-mutant plants. EAST1 encodes the histone acetyltransferase ELONGATA3 (ELO3), a component of the Elongator complex. Genetic analysis, using mutations in genes involved in the biogenesis of a trans-acting small interfering RNA (ta-siRNA), revealed that ELO3 mediated establishment of leaf polarity independently of AS2 and the ta-siRNA-related pathway. Treatment with an inhibitor of histone deacetylases (HDACs) caused additive polarity defects in as2-1 east1-1 mutant plants, suggesting the operation of an ELO3 pathway, independent of the HDAC pathway, in the determination of polarity. We propose that multiple pathways play important roles in repression of the expression of class 1 KNOX and abaxial-determinant genes in the development of the adaxial domain of leaves and, thus, in the establishment of leaf polarity.

Keywords: Arabidopsis thaliana • ASYMMETRIC LEAVES2 (AS2) • ELONGATA3 (ELO3) • Elongator • Histone acetyltransferase • Leaf polarity.

Abbreviations: AS, ASYMMETRIC LEAVES; GFP, green fluorescent protein; HAT, histone acetyltransferase; HDAC, histone deacetylase; KNOX, knotted-like homeobox; MS, Murashige and Skoog; RT–PCR, reverse transcription–PCR; ta-siRNA, trans-acting small interfering RNA; TSA, trichostatin A; UTR, untranslated region

Introduction

Leaves develop, as lateral organs, from the peripheral zone of the shoot apical meristem. Initially, a group of cells, which might be in a determinate state, is generated along the proximal–distal axis. The subsequent establishment of the adaxial–abaxial axis in early-stage leaf primordia is crucial for further leaf development, as cells proliferate along the medial–lateral axis, and results in the flat and symmetrical shape of leaves (Steeves and Sussex 1989, Hudson 2000, Tsukaya 2006).

The PHANTASTICA (PHAN) gene of Antirrhinum majus is involved in the growth and adaxial–abaxial determination of lateral organs and is required early in the establishment of the proximal–distal axis (Waites and Hudson 1995, Waites et al. 1998). The PHABULOSA (PHB), PHAVOLUTA (PHV) and REVOLUTA (REV) genes, which encode class III homeodomain-leucine zipper (HD-ZIPIII) proteins, determine adaxial cell fate (McConnell and Barton 1998, McConnell et al. 2001, Emery et al. 2003) and are negatively regulated by microRNA165 (mir165) and mir166 (mir165/mir166) (Bao et al. 2004, Mallory et al. 2004). Members of the YABBY (YAB) family, such as the
FILAMENTOUS FLOWER (FIL) gene, and of the KANADI (KAN) family, which are involved in the specification of abaxial cell fate in the leaf lamina, have also been identified (Bowman and Smyth 1999, Sawa et al. 1999, Siegfried et al. 1999, Eshed et al. 2001, Kerstetter et al. 2001, Kumar et al. 2002, Eshed et al. 2004, Wu et al. 2008, Sarojam et al. 2010). In addition, ETI1N/AUXIN RESPONSE FACTOR 3 (ETT/ARF3) and ARF4 specify both abaxial cell fate and lateral growth (Pekker et al. 2005). Thus, the mediolateral growth of the lamina is related to the determination of adaxial–abaxial identity (Waites and Hudson 1995, Eshed et al. 2004, Golz et al. 2004). Levels of transcripts of both ETT/ARF3 and ARF4 are regulated by a set of transacting small interfering RNAs (ta-siRNAs) that are known as tasiR-ARFs. These tasiR-ARFs are derived from non-coding TAS3 transcripts, which are initially targeted for cleavage by miR390 and a TAS3-specific small RNA-processing protein, ARGONAUTE7 (AGO7)/ZIPPY. Then the tasiR-ARFs are amplified as a consequence of the activities of the products of SUPPRESSOR OF GENE SILENCING3 (SGS3) and RNA-DEPENDENT RNA POLYMERASE6 (RDR6) and, finally, they are processed by the products of DICER-LIKE4 (DCL4) (Allen et al. 2005, Williams et al. 2005, Xie et al. 2005, Adenot et al. 2006, Faulgren et al. 2006, Hunter et al. 2006, Nagasaki et al. 2007, Nogueira et al. 2007). AGO7 and TAS3 are expressed on the adaxial side of leaf primordia, ensuring that ETT and ARF4 transcripts are restricted to the abaxial side of the primordia (Montgomery et al. 2008, Chitwood et al. 2009, Schwab et al. 2009).


Many mutations, such as mutations in genes for ribosomal proteins, for components of the 26S proteasome, and for biogenesis of tasiR-ARFs, modify or enhance leaf adaxial–abaxial patterning on the as1-1 or as2-1 genetic background (Xu et al. 2003, Li et al. 2005, Garcia et al. 2006, Huang et al. 2006, Xu et al. 2006, Yang et al. 2006, Pinon et al. 2008, Yao et al. 2008, Horiguchi et al. 2011, Szakony and Byrne 2011). Mutations in the chromatin-remodeling gene PICKLE, the HIRA gene for a histone chaperone and the HDT1 and HDT2 genes for histone deacetylases (HDACs) also modify the phenotype of as1/as2 mutant plants (Ori et al. 2000, Phelps-Durr et al. 2005, Ueno et al. 2007), indicating that modifications of chromatin play an important role in leaf development. Thus, it appears that multiple pathways regulate leaf development on the as1 or as2 background, but the roles of the various genes and the mutual interactions among their products during leaf development are unknown.

In the present study, we identified two enhancers of the abnormal leaf development phenotype associated with as2, namely east1-1 and east1-2. Mutations in these enhancers correspond to mutations in the ELONGATA3 (ELO3) gene, which encodes a homolog of Elp3 of yeast and animals. Mutations in ELO3 increased the adaxial defects of leaves on the as2 and as1 backgrounds. The Elongator complex was first identified as a factor that associates with hyperphosphorylated RNA polymerase II in yeast (Otero et al. 1999). Subunits of the Elongator complex, designated Elp1–Elp6, have been purified from yeast, human and plant cultured cells (Wittsieben et al. 1999, Hawkes et al. 2002, Winkler et al. 2002, Nelissen et al. 2010). Previous studies showed that the complex promotes transcription via acetylation of histone H3 and histone H4 (Kristjuhan et al. 2002, Kouskouti and Talianidis 2005, Han et al. 2008, Nelissen et al. 2010), and maintains gene silencing and genome stability during DNA replication in yeast (Li et al. 2009). Mutations in the components of the Elongator complex, encoded by ELO2/ABA-OVERLY SENSITIVE 1 (ABO1)/AtElp1, AtElp2, ELO3, ELO1/AtElp4, AtElp5, AtElp6 and the putative regulator DEFORMED ROOTS AND LEAVES1 (DRL1)/ELO4 in Arabidopsis thaliana, have pleiotropic effects, delaying growth, inducing the formation of narrow leaves and increasing sensitivity to ABA (Nelissen et al. 2003, Nelissen et al. 2005, Chen et al. 2006, Falcone et al. 2007, Zhou et al. 2009, De Fraia et al. 2010). We propose that the Elongator complex regulates the adaxial–abaxial patterning of leaves, acting in concert with the products of the AS1 and AS2 genes in A. thaliana.

**Results**

**Identification of genes for enhancers of as2-mediated defects in leaf development**

In a genetic screening for enhancers and suppressors of the as2-1 mutation, we identified a number of mutations that modified the phenotype of as2-1 leaves (Fig. 1). However, we failed to isolate any suppressors of the as2-1 mutation. We analyzed two independently isolated mutants with similar phenotypes in terms of leaf morphology; each had filamentous leaves and trumpet-shaped leaves. Allelism analysis demonstrated that the two mutants had mutations at the same locus. We named the
The east1-1 and east1-2 mutations enhance the adaxial abnormality of as2-1 leaves. (A) Gross morphology of as2-1. (Ba) Gross morphology of as2-1 east1-1. Arrowheads indicate filamentous leaves. (Bb) Magnified view of Ba, showing filamentous leaves of as2-1 east1-1. (Ca) Gross morphology of as2-1 east1-2. (Cb) Magnified view of Ca, showing filamentous leaves and trumpet-shaped leaves of as2-1 east1-2. (D) Wild-type plant (Col-0). (E) east1-1. (F) east1-2. (G) as1-1. (H) as1-1 east1-1. (I–L) Cotyledons and the first to the 10th [or 11th for Col-0 (K)] rosette leaves of as2-1 (I) and as2-1 east1-1 (Ja). Magnified view of trumpet and filamentous leaves (Jb) and arrays of Col-0 (K) and east1-1 (L) leaves. Plants shown in A–H were photographed 21 d (A), 27 d (B–F), 28 d (G) and 42 d (H) after sowing. White arrowheads and arrows indicate filamentous leaves and trumpet-shaped leaves, respectively.

The east1-1 and east1-2 mutations enhance the adaxial abnormality of as2-1 leaves. (A) Gross morphology of as2-1. (Ba) Gross morphology of as2-1 east1-1. Arrowheads indicate filamentous leaves. (Bb) Magnified view of Ba, showing filamentous leaves of as2-1 east1-1. (Ca) Gross morphology of as2-1 east1-2. (Cb) Magnified view of Ca, showing filamentous leaves and trumpet-shaped leaves of as2-1 east1-2. (D) Wild-type plant (Col-0). (E) east1-1. (F) east1-2. (G) as1-1. (H) as1-1 east1-1. (I–L) Cotyledons and the first to the 10th [or 11th for Col-0 (K)] rosette leaves of as2-1 (I) and as2-1 east1-1 (Ja). Magnified view of trumpet and filamentous leaves (Jb) and arrays of Col-0 (K) and east1-1 (L) leaves. Plants shown in A–H were photographed 21 d (A), 27 d (B–F), 28 d (G) and 42 d (H) after sowing. White arrowheads and arrows indicate filamentous leaves and trumpet-shaped leaves, respectively.

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Table 1 Frequency of abnormal leaves in single and double mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>No. of plants examined</th>
<th>No. and percentage of plants with</th>
<th>No. and percentage of plants with</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>trumpet-shaped leaves</td>
<td>filamentous leaves</td>
</tr>
<tr>
<td>as2-1</td>
<td>80</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>east1-1</td>
<td>49</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>as2-1 east1-1</td>
<td>65</td>
<td>53 (82%)</td>
<td>62 (95%)</td>
</tr>
<tr>
<td>as1-1</td>
<td>88</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>as1-1 east1-1</td>
<td>48</td>
<td>25 (61%)</td>
<td>28 (68%)</td>
</tr>
</tbody>
</table>

Since the as2-1 east1-2 mutant rarely produced flowers and seeds, we focused on the east1-1 mutation in subsequent analysis. The development of vein networks is less extensive in cotyledons and leaves of the as2-1 mutant than in the wild.
type (Semiarti et al. 2001). We also examined venation patterns in the cotyledons and first two leaves of as2-1 east1-1 and corresponding single-mutant plants. The east1-1 mutation had slight defects in patterns of leaf venation. However, the as2-1 east1-1 leaves had more severely defective formation of leaf veins than the single as2-1 and east1-1 mutant leaves (Supplementary Fig. S1).

The as2-1 east1-1 mutant was defective in the establishment of the adaxial cell fate in leaves

To examine the effect of the east1-1 mutation on the adaxial–abaxial polarity of leaves, we used a fusion construct with the gene for green fluorescent protein (GFP) under the control of the Fil promoter (Filp:GFP), which is expressed in abaxial cells of leaf primordia (Watanabe and Okada 2003). When we examined the as2-1 shoot apex from the adaxial side of leaves, we detected a faint signal due to GFP at the edges of developing young leaves (Fig. 2A). We then detected strong signals due to GFP on the abaxial side in sections of leaf primordia (Fig. 2B). When we examined the as2-1 east1-1 shoot apex from the adaxial side of leaves, we detected strong signals at the edges and over the entire surface of serrated leaf blades and petioles (Fig. 2C) and on the entire surface of the filamentous leaves at the fourth and fifth positions (Fig. 2D).

To evaluate the effects of the east1-1 mutation on gene expression, we performed real-time reverse transcription–PCR (RT–PCR) using RNA from aerial parts of 14-day-old plantlets (shown in Fig. 2E–H) and quantified transcripts of class 1 KNOX genes, which are expressed in the shoot apical meristem and at its periphery in wild-type plants; of HD-ZIP III genes (PHB, PHV and REV), which specify adaxial cell fate; and of three families of transcription-related genes, namely YAB genes, KAN genes and ARF genes, which separately and redundantly specify abaxial cell fate. As shown in Fig. 2I, levels of transcripts of a number of genes that are involved in the establishment of abaxial cell fate were higher in the as2-1 east1-1 double mutant than in the wild-type and corresponding single-mutant plants. However, levels of HD-ZIP III transcripts were not significantly different. These results suggested that the filamentous leaves of as2-1 east1-1 plants might have accentuated abaxialized characteristics.

Fig. 2. Filamentous leaves of as2-1 east1-1 were abaxialized. (A) Signals from GFP in the shoot apex of as2-1 that harbored the Fil promoter:GFP (Filp:GFP) construct. Signals due to GFP were observed from the adaxial side. An arrow and arrowheads indicate developing young leaf and flower buds, respectively. Plants shown in A–D were photographed 21 d after sowing. (B) Signals due to GFP (green) and Chl autofluorescence (red) in sections of leaf primordia of as2-1 Filp:GFP. (C) Signals due to GFP in the shoot apex of as2-1 east1-1 that harbored the Filp:GFP construct. An arrow and arrowheads indicate developing young leaf and flower buds, respectively. (D) Signals due to GFP in a transverse section of a filamentous leaf in as2-1 east1-1 Filp:GFP. (E–H) Plants used for the expression analysis in I: Col-0 (E), as2-1 (F), east1-1 (G), as2-1 east1-1 (H). (I) Expression analysis by quantitative real-time RT–PCR. Levels of expression relative to those in wild-type plants (Col-0) are shown in the histogram. Results were normalized by reference to levels of ACTIN2 transcripts. Total RNA was extracted from above-ground parts of seedlings 14 d after sowing.
Accumulation of KNOX transcripts was also greater in the as2-1 east1-1 double mutant than in the wild type, and as2-1 and east1-1 single-mutant plants. These results suggest that as2-1 east1-1 leaf cells are in a more indeterminate and more abaxialized state than the wild-type leaf cells.

**The east1-1 and east1-2 mutations were mapped to the ELONGATA3 gene**

The locus responsible for the east1 phenotype was located between 20.1 and 21.7 Mb on chromosome V, as determined from an analysis of 533 F2 segregants. The site of the east1-1 mutation was mapped to a 50 kb region of the genomic DNA. Sequence analysis revealed that the east1-1 and east1-2 mutations corresponded to one-base and two-base substitutions, respectively, in At5g50320 (gene code of the Arabidopsis Genome Initiative), namely ELONGATA3 (ELO3) (Fig. 3A). The ELO3 and ELP3 proteins of other eukaryotes include an N-terminal domain that is conserved in the radical S-adenosylmethyltransferase superfamily (radical SAM domain) and a C-terminal G(N/A)T-type histone acetyltransferase (HAT) domain that is related to GCN5 (Wittschieben et al. 1999, Pandey et al. 2002; Supplementary Fig. S2).

In the east1-1 and east1-2 mutants, levels of the corresponding mRNAs were the same as in the wild-type plants (Fig. 3C). However there was one type of east1-1 mRNA and four types of east1-2 mRNA (Fig. 3B). In the product of east-1, we found that a conserved arginine residue had been changed to a tryptophan residue in the radical SAM domain (Fig. 3A, Supplementary Fig. S2). The east1-2 mutant had two independent base substitutions in the ELO3 gene; one caused replacement of a non-conserved alanine residue by a glycine residue in the radical SAM domain (Fig. 3A); the other was a single base change (G to A) at the 5' splice site of the fourth intron, which resulted in four classes of abnormally spliced transcripts (east1-2a, -2b, -2c and -2d; Fig. 3B, C). The major transcript in east1-2 plants (Fig. 3B, C), designated east1-2a, included the non-spliced fourth intron, which caused the mis-translation of 61 amino acid residues and a stop codon within the radical SAM domain. Minor splicing variants in east1-2 plants, designated east1-2b through east1-2d, also included the respective internal stop codons within the radical SAM domain (indicated by asterisks in Fig. 3B). In east1-2b, the fourth exon lacked 8 bp at the 3' end and was joined to the fifth exon, with a resulting premature stop codon in the fourth exon. The east1-2c transcript included a 12 bp sequence at the 3' end of the third intron and the 93 bp fragment at the 5' end of the fourth intron. The east1-2d transcript included the 67 bp fragment at the 5' end of the fourth intron, and a frameshift created a stop codon in the fifth exon. All proteins predicted from the various east1-2 mutant mRNAs appeared to lack the HAT domain. These results are consistent with the observation that as2-1 east1-2 plants had more severe defects than as2-1 east1-1 plants, which generated the full-length protein with a single amino acid substitution in the radical SAM domain.

We performed a complementation test using as2-1 east1-1 double mutants and a 5.6 kb genomic fragment that contained the ELO3 coding region; a 0.5 kb upstream region, which contained part of the 3'-untranslated region (UTR) of an adjacent gene; and a 1 kb 3'-downstream region. An as2-1 east1-1 transformant harboring the ELO3 genomic fragment had the as2-1 single-mutant phenotype (Fig. 3D). T2 progeny also had a
typical as2-1 single-mutant phenotype in the presence of the transgene. We concluded that mutations in ELO3 were responsible for the enhanced mutant phenotype of the as2-1 east1-1 double mutant plants.

**Mutation of the gene for another component of the Elongator complex affected establishment of leaf adaxial–abaxial polarity**

To investigate the relationship between AS2 and the Elongator complex, we examined whether other elongata mutations might result in enhanced defects in adaxial–abaxial polarity on the as2-1 background. First, we produced the double mutant as2-1 elo2-1 with the Ler genetic background, by crossing elo2-1 (Ler) with an as2-1 plant backcrossed twice with Ler. The as2-1 elo2-1 mutant had filamentous leaves (Fig. 4B). Secondly, we produced an as2-1 elo2-3 mutant by crossing as2-1 (Col-0) with an elo2-3 plant, which is a T-DNA insertion line with the Col-0 background (Nelissen et al. 2005). The as2-1 elo2-3 mutants produced trumpet-shaped leaves (Fig. 4C). The incomplete effect of elo2 on the Col-0 background has been described previously (Nelissen et al. 2005).

The elo4 mutation and the weak allele dri1, also caused weakly defective adaxial–abaxial polarity on the as2-1 background. We produced as2-1 elo4 plants with the Ler background, by crossing elo4 (Ler) with an as2-1 plant backcrossed twice with Ler. The as2-1 elo4 plant had trumpet-shaped leaves (Fig. 4E). The as2-1 elo4 plants backcrossed twice with Col-0 had somewhat trumpet-shaped or narrow downwardly curling leaves (Fig. 4F). These results suggested that the Elongator complex might be required for establishment of the adaxial–abaxial polarity of leaves.

**Fig. 4** Mutations in genes for various components of the Elongator complex enhanced adaxial–abaxial defects of as2-1 leaves. (A) Gross morphology of the elo2-1 mutant with the Ler background (Ler). (B) as2-1 elo2-1 (Ler). (C) as2-1 elo2 with the Col-0 background (Col-0). (D) elo4 (Ler). (E) as2-1 elo4 (Ler). (F) as2-1 elo4 (Col-0). All plants shown were photographed 27–28 days after sowing. White arrowheads and arrows indicate filamentous leaves and trumpet-shaped leaves, respectively.

Mutations in ELO3 and treatment with an HDAC inhibitor caused additive damage to the adaxial development of as2 leaves

In a previous study, we showed that inhibition of HDACs by trichostatin A (TSA), a specific inhibitor of HDACs, on the as2-1 background causes severe defects in the adaxial development of leaves, with efficient generation of abaxialized filamentous leaves (Ueno et al. 2007). To investigate the relationship between ELO3 and genes for HDACs, we treated wild-type (Col-0), as2-1, east1-1 and as2-1 east1-1 plants with 1 or 3 μM TSA for 2 weeks. In the absence of TSA, wild-type and as2-1 plants did not produce any filamentous leaves (Fig. 5A, B) and the as2-1 east1-1 double mutant also did not produce any filamentous leaves for 2 weeks (Fig. 5D). Upon treatment with 1 μM TSA, wild-type plants exhibited delayed growth but did not produce filamentous leaves (Fig. 5E). However, 29% of as2-1 plants, 4% of east1-1 plants and 76% of as2-1 east1-1 plants produced filamentous leaves (Fig. 5F–I). Thus, treatment with TSA of as2-1 east1-1 plants stimulated the production of filamentous leaves in response to the double mutation, as compared with single mutations, and TSA caused the more efficient production of filamentous leaves in as2-1 east1-1 plants than was observed in the absence of treatment. Wild-type plants treated with 3 μM TSA exhibited significantly delayed growth (Fig. 5J), but only 4% generated filamentous leaves. In contrast, 86% of as2-1 plants, 14% of east1-1 plants and 28% of as2-1 east1-1 plants had filamentous leaves after treatment with 3 μM TSA (Fig. 5K–O). The lower frequency of filamentous leaves in as2-1 east1-1 plants at 3 μM TSA might have been due to the severely defective growth of these plants. In fact, 17% of east1-1 plants and 64% of as2-1 east1-1 plants failed to develop any true leaves at 3 μM TSA (Fig. 5M, N, P). Thus, both treatments with TSA caused marked additional defects in the leaf polarity and growth of as2-1 east1-1 plants.

**Mutations in ELO3 and RDR6 or AGO7 caused additive damage to the adaxial development of as2 leaves**

We investigated the effects of the tasiR-ARF pathway on polarity defects in east1-1 and as2-1 east1-1 plants using the rdr6 and ago7 mutations. RDR6 and AGO7 are involved in the biogenesis of trans-acting siRNA, known as tasiR-ARF, that targets ETT and ARF4 mRNAs (Allen et al. 2005, Adenot et al. 2006, Faulgren et al. 2006, Hunter et al. 2006). rdr6-30 and ago7-1 mutants have a similar downward-curling leaf phenotype (Vazquez et al. 2004, Fig. 6D, G). Our as2-1 rdr6-30 and as2-1 ago7-1 plants had narrower cotyledons and more serrated rosette leaves than the wild-type and the as2 plants, as reported previously (Fig. 6E, H; Li et al. 2005, Garcia et al. 2006, Xu et al. 2006). The double mutants east1-1 rdr6-30 and east1-1 ago7-1 exhibited a more severe phenotype with more downwardly curling and narrower leaves than the single mutants (Fig. 6Fa, 1a). The fifth or sixth leaves often developed two tandem leaf blades on a single petiole (Fig. 6Fb, Fc, 1b).
The triple mutant as2-1 east1-1 rdr6-30 had even more severe defects in leaf development than the as2-1 east1-1 and as2-1 rdr6-30 double mutants (Fig. 6Ja): plants were significantly reduced in size and developed very narrow cotyledons, only a few leaves with very small leaf blades, and needle-like leaves (Fig. 6Jb), but no reproductive organs. The observed leaf phenotype appeared to reflect enhanced defects in the establishment of adaxial–abaxial polarity.

Expression of 34 genes was enhanced in as1 and as2 mutants and in plants with mutations in genes for components of the Elongator complex

To identify genes whose expression might be controlled by ELO3, we applied the knowledge-based fuzzy adaptive resonance theory (KB-FuzzyART; Takahashi et al. 2008) to the clustering of profiles of gene expression that were obtained by microarray analysis (with the Affymetrics GeneChip) of mRNAs from wild-type Arabidopsis (Ler), elo2-1, elo2-1 and drl1-2 plants (Nelissen et al. 2005). Since the results in the previous section (Fig. 4) indicated that mutations in genes (drl1, elo2 and elo3) for various subunits and regulators of the Elongator complex had similar effects on polarity development in as2 plants, we looked for genes whose levels of expression were altered similarly in the various mutants when compared with wild-type levels. We identified 851 candidate genes. As shown in Fig. 7A and Supplementary Table S1, we were able to classify these 851 genes by KB-FuzzyArt into clusters I–V, and 45 outliers. These 851 genes did not contain the AS1 and AS2 genes.

Clusters III, IV and V included genes whose expression was suppressed in the three mutants. Cluster III included the IAA13 gene and cluster IV included IAA3 (SHY2), a result that is consistent with the result published by Nelissen et al. (2010). Cluster V included genes, such as IAA5 and SAUR, that are known as auxin-inducible genes. Clusters I and II included genes whose expression was enhanced in the three mutants, and the STM gene was categorized to cluster II, consistent with a previously published result (Nelissen et al. 2010). Cluster III included six genes for histones H2A, H2B, H3 and H4, whose...
expression was strongly suppressed in the mutants. It seems that ELO3 might be involved in the assembly of chromatin during the S phase.

Clusters I and II (Fig. 7B) included 371 genes whose expression was enhanced in elo3-1, elo2-1 and drl1-2 plants. We previously published the results of cluster analysis of gene expression in as2-1 and as1-1 plants and the overexpressor of AS2 (Takahashi et al. 2008). Using our new list of genes for cluster analysis (Supplementary Table S2), we obtained 18 refined clusters. The ELO2, ELO3 and DRL1 genes showed no significant changes in microarray data profiles of wild-type, as2-1 and as1-1 plants. We identified 381 genes that belonged to two clusters, clusters 3 and 6, whose expression was enhanced both in as2-1 and as1-1 plants and suppressed in the overexpressor of AS2. Clusters 3 and 6 included the BOP and KAN2 genes, respectively. We observed that expression of 34 genes was enhanced in as1-, as2- and elo3-related mutants (Fig. 7B, Supplementary Table S3). These genes included STM, a meristem-specific gene, as well as LSH3 and LSH4, which are expressed at the boundaries between the shoot apex and leaf primordia (Cho and Zambryski 2011, Takeda et al. 2011).

**Discussion**

In the present study, we found that as2-1 east1-1 double-mutant of *A. thaliana* plants formed filamentous and severely serrated leaves with an abaxialized surface (Figs. 1, 2), and our results suggest that the ELO3 gene is involved in adaxial development on the as2 background. The as2 and elo3/east1-1 mutations resulted in synergistic increases in levels of transcripts of many abaxial-determinant genes, such as *ETT*/*ARF3*, *FIL* and *YAB5* and all four class 1 KNOX genes (Fig. 2), some of which have been identified as acting downstream of AS2 (Semiarti et al. 2001, Iwakawa et al. 2007). Furthermore, our results suggest an essential role for the Elongator complex, which includes...
ELO3 and other subunits, in the establishment of adaxial polarity on the as2 background.

The Elongator complex is involved in repression of abaxial-determinant and class 1 KNOX genes through an as yet unidentified pathway that acts in parallel with the AS2/AS1 pathway

There are three possible models to explain how AS2/AS1 and the Elongator complex might be related to each other in the establishment of the adaxial–abaxial polarity of leaves in wild-type A. thaliana. In the first model, the Elongator complex might act on adaxialization independently of AS2/AS1 during leaf development: each might regulate common target genes to control the adaxialization of a leaf via two parallel pathways. It has been reported that the AS2/AS1 complex represses transcription of the BP and KNAT2 genes directly (Guo et al. 2008). Transcription of ETT is also repressed by AS1 and AS2 (Iwakawa et al. 2007). Our expression analysis revealed that levels of BP, KNAT2, and KNAT6 transcripts were slightly higher in as2 than in the wild type, consistent with previous results (Semiarti et al. 2001, Iwakawa et al. 2007, Guo et al. 2008) and that the level of the STM transcript was slightly elevated in east1-1 plants and as1 leaves (Fig. 2; Semiarti et al. 2001). In addition, levels of transcripts of all four class 1 KNOX genes were markedly elevated in as2-1 east1-1 double-mutant plants (Fig. 21). The levels of the transcript of the FIL gene was slightly elevated in as2 or elo3/east1-1 mutant plants, but markedly elevated in as2 elo3/east1-1 double-mutant plants. It has been reported that AS1 represses expression of the FIL gene (Garcia et al. 2006). These observations suggest that the Elongator complex might somehow repress the expression of the class 1 KNOX and FIL genes via an unidentified pathway that might operate in parallel with the AS2/AS1-containing pathway (Fig. 8), but we do not yet know whether the Elongator complex controls transcription of these genes directly or indirectly.

In a second possible model, AS2 and the Elongator complex might play biochemically redundant roles in repression of the expression of class 1 KNOX and abaxial-determinant genes. Since there are no similarities in terms of amino acid sequences between AS2 and ELO3, this possibility seems unlikely.

In a third possible model, AS2/AS1 and the Elongator complex might act via two distinct respective pathways, which might control functionally distinct genes and/or proteins. However, the observed severe defects in the adaxialization of leaf development in the as2 elo3/east1-1 double mutant might be due to the synthetic phenotypes of distantly related (or unrelated) phenotypes that might have been generated independently by as2 and elo3/east1-1. Levels of transcripts of class 1 KNOX genes, such as BP and STM, and levels of FIL transcripts did, however, increase similarly in the as2 and elo3/east1-1 single mutants, and the double mutation clearly raised the levels of transcript of these genes. The similar alterations in patterns of gene expression in as2 and elo3/east1-1 single mutants and in the double mutant suggest that this third possibility might be unlikely.

The first model provides the simplest explanation of the way in which the AS2/AS1 and the Elongator complex might be involved in the repression of the expression of class 1 KNOX and abaxial-determinant genes (Fig. 8). In addition, wild-type AS2/AS1 appears to complement potential defects in gene expression generated by mutations in the Elongator complex, as discussed below.

How might the Elongator complex repress expression of abaxial-determinant and class 1 KNOX genes?

The ELO3 gene encodes a homolog of yeast Elp3, which has HAT activity, and ELO3, and yeast and mammalian Elp3 proteins are involved in promotion of the transcription of a wide range of genes (Kouskouti and Talianidis 2005, Nelissen et al. 2005, Han et al. 2007, Han et al. 2008, Nelissen et al. 2010). The elo3-6 mutation of Arabidopsis causes a decrease in levels of acetylation of histone H3K14 in the coding region and 3’-UTRs of the SHY2/IAA3 and LAX2 genes (Nelissen et al. 2010), suggesting that histone acetylation by wild-type ELO3 might allow transcription of these genes. In the present study, the Elongator complex appeared to repress transcription of abaxial-determinant and class 1 KNOX genes. It is unlikely that the Elongator complex might directly repress such gene expression via histone acetylation. Rather, it is likely that ELO3 might positively control transcription of an unidentified gene whose product might then repress expression of these genes.

In addition to its role in the activation of transcription, yeast Elp3 has been reported to be involved in transcriptional silencing in telomeric regions and in the maintenance of genome
In the present study, we showed that as2 elo3/east1-1 development of multiple pathways are involved in the adaxial–abaxial determination and class 1 KNOX genes. We speculate that some factors involved in the establishment of adaxial polarity of leaves. In addition, triple mutations, such as as2 elo3/east1-1 rdr6, resulted in more severe defects in polarity than double mutations, such as as2 rdr6, as2 ago7 and as2 elo3/east1. The RDR6 and AGO7 genes are involved in the single pathway that is involved in the biogenesis of tasiR-ARF, which is involved in the degradation of ETT and ARF4 transcripts (Allen et al. 2005, Williams et al. 2005, Adenot et al. 2006, Montgomery et al. 2008, Chitwood et al. 2009). Our genetic analysis suggests that the process mediated by ELO3 is distinct from the tasIR-ARF pathway.

Treatment of the as2 elo3/east1-1 double mutant with TSA enhanced the polarity defects of the mutant (Fig. 5). Genes for two TSA-sensitive HDACs that are related to the establishment of leaf polarity have been identified as HDT1 and HDT2 (Ueno et al. 2007). The observed additive effect of TSA on the polarity defect of the as2 elo3/east1-1 double mutant suggests that HDT1/HDT2 acts independently of the ELO3 pathway. Thus, the adaxial development of leaves might be controlled independently by multiple pathways that include the products of ELO3, RDR6/AGO7 and HDT1/HDT2 on the as2 background.

Our genetic analysis showed that the wild-type AS2/AS1 replacement replaces the polarity establishment function of the ELO3, RDR6 (AGO7) and HDT1/HDT2 genes. When AS2 is functional, adaxial defects due to east1-1, rdr6/ago7 and hdt1/hdt2 monogenic mutations are not obvious (Fig. 6). Alterations in expression of abaxial-determinant and class 1 KNOX genes are also ambiguous in these cases (Fig. 2). The adaxialization of leaves is, however, severely disrupted, and expression of abaxial-determinant and class 1 KNOX genes is enhanced, once expression of any one of these genes is disrupted in the absence of AS2 function, even though the molecular events that might be directly governed by these genes (ELO3, RDR6/AGO7 and HDT1/HDT2) do not seem to be closely related to one another.

A wild-type AS2 gene appears to complement deficiencies in the unrelated events mediated by these latter genes. Further investigations of the molecular functions of AS2 should help us to understand more details of the way in which AS2/AS1 might regulate gene expression.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Col-0 (CS1092), and the as1-1 (CS3374), as1-2 (CS3117) and elo2-3/abo1-2 (N504690) mutants were obtained from the Arabidopsis Biological Resource Center (ABRC). We outcrossed as2-1 with Col-0 three times and as1-1 with Col-0 once, and used the progeny for our experiments (Semiarti et al. 2001). For analysis of phenotypes, seeds were sown on soil or on plates of MS (Murashige and Skoog) medium. After 2 or 3 d at 4°C in darkness, plants were transferred to a daily regimen of 8 h of darkness and 16 h of
white light at 50 μmol m⁻² s⁻¹ at 22°C, as described previously (Semiarti et al. 2001). Ages of plants are given in terms of numbers of days after sowing. The elo2-1 and elo4 mutants were in Ler, as described (Berná et al. 1999, Nelissen et al. 2005). The seeds of elo2-1, elo4 and ago7-1 (NS37458, available from the ABRC) were kindly provided by Professor J. L. Micol at the Universidad Miguel Hernández and Professor Y. Watanabe at the University of Tokyo.

Screening of enhancer of as2-1

About 2,000 as2-1 seeds were treated with 0.4% ethylmethane sulfonate (EMS) for 8 h and then these M₁ seeds were germinated on soil. M₂ seeds derived from 13–33 M₁ plants were collected to obtain the 81 stocks of M₂ seeds. At least 100 plants from each stock were grown on the soil or MS agar medium, and the leaves were observed for morphology. Each enhancer mutant was crossed with wild-type Col-0 to generate F₁ seeds. F₂ seeds were grown on the soil and about 5% of the progeny showed a phenotype similar to the original phenotype. We also isolated the east4 mutant, which was a new rdr6 mutant allele, namely rdr6-30, from the population of M₂ seeds (our unpublished data).

Map-based cloning of east1-1

The F₂ population of a cross between east1-1 and as2-5 (Ler) was used for the genetic mapping of east1 with mapping primers as described previously (Tanaka et al. 2004). For complementation, 5.7 kb of a SacI–BstNI genomic fragment was cloned into the binary vector pGreen0029 (Hellens et al. 2000) and transformed into as2-1 east1-1 plants by Agrobacterium-mediated transformation (Bechtold and Pelletier 1998). A kanamycin-resistant T₁ plant with as2-1 phenotype was isolated and T₂ progeny were segregated to kanamycin-sensitive and kanamycin-resistant populations with as2-1 east1-1 and as2-1 phenotype, respectively. Primers for high-resolution mapping and genotyping of the mutations are listed in Supplementary Table S4.

Real-time RT–PCR

Leaves and shoot apices of wild-type and mutant plants were harvested at 14 d after sowing, and immediately frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated by using the RNeasy Plant Mini Kit (Qiagen). For the analysis of RNA levels by real-time PCR, we prepared 5 μg of total RNA to convert to cDNA as described by Iwakawa et al. (2007). PCR was performed in the presence of the double-stranded DNA-specific dye Power SYBR Green (Applied Biosystems) with primers as described (Iwakawa et al. 2007). Amplification was monitored in real time by using the Applied Biosystems StepOnePlus Real-Time PCR system, according to the supplier’s recommendations. The mean value of three technical replicates was normalized by the value of ACTIN2 transcripts.

Fluorescence microscopy

We crossed as2-1 east1-1 and as2-1 FILp:GFP (Watanabe and Okada 2003, Ueno et al. 2007). Shoot apices containing leaf primordia were embedded in 7% agarose, and agarose blocks were sliced into thin sections. Fluorescence was observed with an LSM510 confocal laser scanning microscope (Carl Zeiss Inc.).

Microarray data analysis of as1-1, as2-1 and as2-1/pAS1::AS2 mutants

Gene expression data were obtained by using ATH1 Affymetrix chips (Affymetrix) and total RNA from shoot apices of 15-day-old Col-0, as1-1, as2-1 and as2-1/pAS1::AS2 plants as described (Takahashi et al. 2008). In this study, we conducted reanalysis using new list of genes for A. thaliana (Supplementary Table S2). A total of 18 clusters were constructed for the same array data set by KB-FuzzyART (Takahashi et al. 2008). Clusters 3 and 6 among these clusters were merged and used in the following analysis, as shown in Supplementary Table S3.

Microarray data analysis of elo2-1, elo3-1 and drl1-2 mutants

The Affymetrix microarray data for Ler, elo2-1, elo3-1 and drl1-2 mutants (Nelissen et al. 2005) were retrieved from ArrayExpress (E-MEXP-300). Data were obtained from a total of 12 arrays (triplicate experiments for four strains). The raw data were processed by using Affymetrix Gene Chip Operating Software (GCOS; Version 1.4.0.36). Signal intensities were calculated by using perfect match/mismatch probe pairs and scaled to a target intensity of 200 by using GCOS. For these data, it is possible to calculate nine pairs (triplicate experiments of mutant)/(triplicate experiments of wild type) for each set of mutant/wild-type ratios. Therefore, a total of 27 signal ratio data points (9 pairs × 3 mutants) were calculated by using GCOS. The detection calls and change calls were calculated based on the signal intensity ratio between perfect match and mismatch oligos on the array. The detection calls are present, marginal or absent call (i.e. detection call determined by GCOS, based on the P-value of the one-sided Wilcoxon signed-rank test, present call means 0 ≤ P < 0.05, marginal call means 0.05 ≤ P < 0.065 and absent call means 0.065 < P ≤ 1, respectively). The change calls are an increase, no change or decrease call (i.e. change call determined by GCOS, based on the P-value of the one-sided Wilcoxon signed-rank test, increase call means 0 ≤ P < 0.006, no change call means 0.006 ≤ P ≤ (1−0.006) and decrease call means (1−0.006) < P ≤ 1, respectively). In the present study, we used 22,591 genes (excluding probe sets for control and mitochondria) from 22,810 genes on the ATH1 chip. For gene selection, we excluded 5,940 genes for which all 12 samples, showed an absent call, and 7,347 genes for which all 27 pairs showed no change calls. Therefore, we included those genes (6,888 genes).
for which all calls showed the same pattern of calls for each mutant, because the same pattern of calls indicates high reproducibility. Finally, 851 genes were selected and the median signal ratios for each gene were calculated from nine pairs of ratios for each set of mutant/wild type. These data were used for clustering analysis.

Supplementary data

Supplementary data are available at PCP online.

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