Dual regulation of ETTIN (ARF3) gene expression by AS1-AS2, which maintains the DNA methylation level, is involved in stabilization of leaf adaxial-abaxial partitioning in Arabidopsis

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SUMMARY
Leaf primordia are generated at the periphery of the shoot apex, developing into flat symmetric organs with adaxial-abaxial polarity, in which the indeterminate state is repressed. Despite the crucial role of the ASYMMETRIC LEAVES1 (AS1)-AS2 nuclear-protein complex in leaf adaxial-abaxial polarity specification, information on mechanisms controlling their downstream genes has remained elusive. We systematically analyzed transcripts by microarray and chromatin immunoprecipitation assays and performed genetic rescue of as1 and as2 phenotypic abnormalities, which identified a new target gene, ETTIN (ETT)/AUXIN RESPONSE FACTOR3 (ARF3), which encodes an abaxial factor acting downstream of the AS1-AS2 complex. While the AS1-AS2 complex represses ETT by direct binding of AS1 to the ETT promoter, it also indirectly activates miR390- and RDR6-dependent post-transcriptional gene silencing to negatively regulate both ETT and ARF4 activities. Furthermore, AS1-AS2 maintains the status of DNA methylation in the ETT coding region. In agreement, filamentous leaves formed in as1 and as2 plants treated with a DNA methylation inhibitor were rescued by loss of ETT and ARF4 activities. We suggest that negative transcriptional, post-transcriptional and epigenetic regulation of the ARFs by AS1-AS2 is important for stabilizing early leaf partitioning into abaxial and adaxial domains.

KEY WORDS: Arabidopsis thaliana, ASYMMETRIC LEAVES1 (AS1), ASYMMETRIC LEAVES2 (AS2), AUXIN RESPONSE FACTOR3, AUXIN RESPONSE FACTOR4, Gene body methylation, Leaf polarity

INTRODUCTION
Leaves develop as lateral organs from the peripheral zone of a shoot apical meristem. Initially, a group of cells is patterned along the proximal-distal axis and then establishment of the adaxial-abaxial axis is crucial for further leaf development. Subsequent cell proliferation along the medial-lateral axis results in flat and mediolateral symmetric leaves (Steeves and Sussex, 1989; Waites et al., 1998; Hudson, 2000; Byrne et al., 2001; Semiarti et al., 2001; Tsukaya, 2006; Bowman and Floyd, 2008; Szakonyi et al., 2010; Nakata et al., 2012).

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The PHANTASTICA (PHAN) MYB gene of Antirrhinum majus is involved in growth and adaxial-abaxial determination of lateral organs. Its activity is required early in the establishment of the proximal-distal axis (Waite and Hudson, 1995; Waite et al., 1998). The class III HD-ZIP genes of Arabidopsis thaliana specify the adaxial identity of lateral organs (McConnell and Barton, 1998; McConnell et al., 2001; Emery et al., 2003; Bao et al., 2004; Mallory et al., 2004). Members of the KANADI (KAN) and FILAMENTOUS FLOWER (FIL) [also known as YABBY (YAB)] gene families have been identified as abaxial determinants (Bowman and Smyth, 1999; Eshed et al., 1999; Sawano et al., 1999; Siegfried et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001; Bowman and Floyd, 2008; Goldshmidt et al., 2008; Sarojam et al., 2010). Other components determining abaxial cell identity include the AUXIN RESPONSE FACTOR3 (ARF3) [also known as ETTIN (ETT)] and AUXIN RESPONSE FACTOR4 (ARF4) genes (Sessions and Zambrayski, 1995; Sessions et al., 1997; Pekker et al., 2005; Wu et al., 2008; Kelley et al., 2012). Expression of ETT is regulated by several developmental mechanisms at either transcription or translation levels (Nishimura et al., 2005; Ng et al., 2009; Inagaki et al., 2009). In addition, ETT and ARF4 are both targeted by a trans-acting small interfering RNA (ta-siRNA) called tasiR-ARF (Allen et al., 2005; Williams et al., 2005; Fahlgren et al., 2006; Hunter et al., 2006; Nogueira et al., 2006; Nogueira et al., 2007; Schwab et al., 2009; Chitwood et al., 2009) derived from non-coding TAS3 transcripts that are initially targeted for cleavage by miR390. ARGONAUTE7, RNA-DEPENDENT RNA POLYMERASE6 (RDR6) and DICER-LIKE4 are involved in the biogenesis of tasiR-ARF (Peragine et al., 2004; Allen et al., 2005; Gascioli et al., 2005; Xie et al., 2005; Adenot et al., 2006; Montgomery et al., 2008).
The asymmetric leaves (asl) mutant is disrupted in the PHAN MYB ortholog. AS1 forms a complex with ASYMMETRIC LEAVES2 (AS2) (Guo et al., 2008; Yang et al., 2008) (referred to herein as AS1-AS2). Mutations in AS1 and AS2 have similar pleiotropic effects: asymmetric leaves along the leaf margin, downwardly curled leaves, malformed vein systems with a less-prominent mid-vein, and ability to regenerate increased in shoots but decreased in roots (Rédei and Hirono, 1964; Byrne et al., 2000; Semiarti et al., 2001). Mutations in either as1 or as2 are enhanced by mutations in the ta-siRNA biogenesis pathway to produce leaves with an abnormal mediolateral axis and abaxialized characteristics (Kojima et al., 2011). In addition, chromatin modification, cell proliferation and ribosomal proteins modify or enhance leaf adaxial-abaxial patterning in the as1 or as2 genetic background (Kojima et al., 2011; Horiguchi et al., 2011; Xu et al., 2012; Ishibashi et al., 2012; Nakagawa et al., 2012), suggesting that several pathways apparently regulate leaf development in the as1 or as2 background. Furthermore, transcript levels of class 1 KNOX genes and some abaxial-identity genes are increased in as1 and as2 (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Iwakawa et al., 2007; Ikezaki et al., 2010), indicating that AS1 and AS2 might act as upstream regulators of these genes, although such a mechanism is yet unknown.

AS1 encodes a myb (SANT) domain protein and AS2 encodes a nuclear protein that includes the AS2/LOB domain and belongs to the AS2/LOB family (Byrne et al., 2000; Iwakawa et al., 2002; Shuai et al., 2002; Matsumura et al., 2009). Both AS1 and AS2 transcripts accumulate in the early stage of above-ground organ (CS3374), Details of Col-0 (CS1092), and MET1-1 (CS11092) (referred to CS3374), and the T7 antibody data set (T7 antibody versus mock) were analyzed by Tiling 1.0R arrays (Affymetrix). Scanning was performed at 3.8× magnification and with a pixel size of 2 μm. The image data were analyzed using the Affymetrix GeneChip Operating System (Affymetrix, Santa Clara, CA, USA) and the Affymetrix GeneChip Analysis Software (Affymetrix, Santa Clara, CA, USA). The microarray data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/).

MATERIALS AND METHODS

Plants and plasmid construction

Details of Col-0 (CS1092), as1-1 (CS3374), as2-1 (CS3117) and adr6-10 were described by Kojima et al. (Kojima et al., 2011) and Ishibashi et al. (Ishibashi et al., 2012); ett-13 and arf4-1 were described by Pekker et al. (Pekker et al., 2005); and met1-1 was described by Kankel et al. (Kankel et al., 2003). Plants were grown on Murashige and Skoog (MS) medium with and without demethasone (Sigma-Aldrich, St Louis, MO, USA), cycloheximide (Wako Pure Chemicals, Osaka, Japan), mifepristone (RU486) (Sigma-Aldrich) or 5-aza-2′-deoxycytidine (Sigma-Aldrich). To generate the ETT promoter-GUS construct, the 4.9-kb pETTAMAR-GUS plasmid (herein after designated as pETT4.9kb-GUS) (Ng et al., 2009) was digested with NotI and XbaI, filled with the Klenow fragment of DNA polymerase, and self-ligated to generate pETT2.3kb-GUS. Schematic representation of pAS1:TT7:AS1 and pAS2:AS2:FLAG is shown in supplementary material Fig. S1A. To generate the DNA construct for expression of T7-tagged AS1 expression under the control of the AS1 promoter, we amplified the amino-terminal coding sequence of the AS1 gene by PCR with primers T7F1SalI (5′-CTGTTCAAGGCCGATCTGGCTGCATTGACTACAAGGATGACGATGACAAGCCCGGG-3′) and AS1R1NotI (5′-GAGGGCCCGCGTCAGGGGCGGCTCAGCAATCTGGAAC-3′) with pBSK-AS1 (Iwakawa et al., 2002) as a template. We then inserted the amplified sequence between the EcoRI and NotI sites of pBSK-AS1. The resultant fusion construct was cloned into the pGreen0029 binary vector (Hellens et al., 2000) to yield pAS1:TT7:AS1. The coding DNA sequence from which the translation codon had been deleted was fused to the sequence for three tandem FLAG tags that was amplified by PCR with primers pU330-FLAG5′-CTAGGACTACAAGGATCAGATGGGATATTTAAAGACCCACGATCATGCACTAAAGGATGACGATGACAAGCCGGGG-3′ and pU331-FLAGA (5′-GATCCCCCGGGCGTTCATGCTGACTTCTGTTGATGCAATCTGGAAC-3′). After digestion with BglII, the fusion construct was isolated and inserted between the BglII and NotI sites of pBS35SAS2YPF (Ueno et al., 2000). The resultant plasmid was designated pAS2:3xFLAG. The fragment containing the AS2:3xFLAG gene was amplified from pAS2:3xFLAG by PCR with primers AS2F1SalI (5′-GAGGGCCCGCGTCAGGGGCGGCTCAGCAATCTGGAAC-3′) and FLAGR2NotI (5′-AACAAACTCAC-3′) as a template. We then inserted the amplified sequence between the As1 and Sall sites of pSa2-TAS2, which was constructed by the insertion of the Sall of AS2 (nucleotides −3301 to −1), the recognition sites of As1 and Sall, and the 3′ region of AS2 (nucleotides +595 to +2868), into the ApaI/EcoRV sites of pGreen0029 (Hellens et al., 2000). The resulting plasmid was designated pAS2:3xFLAG. Construction of p35S::GFP:AS1 and p35S:AS2:GR was described previously (Ueno et al., 2007). The histochemical assay for β-glucuronidase (GUS) activity was described previously (Iwakawa et al., 2007).

Production of antibodies

Antibodies against AS1 were prepared by immunizing rabbits with the synthetic peptide RLTKFLEQQMGCRLDRP (residues 537 to 573 of AS1) as antigen. Antibodies were affinity-purified with immobilized antigen, which was prepared by using the SulfoLink Immobilization Kit for Peptides (Thermo Scientific, Waltham, MA, USA). Antibodies specific for T7 (Millipore, Billerica, MA, USA), FLAG (Agilent Technologies, Santa Clara, CA, USA) and GFP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for immunoprecipitation.

ChIP-Chip and ChiP-PCR analyses

Chromatin immunoprecipitation (ChIP) was performed as described by Ng et al. (Ng et al., 2009). For ChIP-chip, chromatin was prepared from as1-1 seedlings containing pAS1:TT7:AS1, and fragmented chromatin was immunoprecipitated with T7-specific and AS1-specific antibodies. The fragmented chromatin was also precipitated without serum, and the resultant precipitates were used as mock controls. Fragmentation and labeling of amplified samples were performed with the GeneChip WT Double-Stranded DNA Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA). Hybridization, staining and washing were performed with GeneChip Arabidopsis Tiling 1.0R arrays (Affymetrix). Scanning was performed at 5.7× magnification with a GeneChip Scanner 3000 7G system. Arabidopsis Tiling 1.0R arrays, the AS1 antibody data set (AS1 antibody versus mock), and the T7 antibody data set (T7 antibody versus mock) were analyzed by the Affymetrix GeneChip Command Console. Raw CEL data files obtained from tiling array experiments were analyzed with Tiling Analysis Software.
ARRAY STUDY
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(TAS Version 1.1.02, Affymetrix). Array data have been deposited in the NCBI Gene Expression Omnibus under accession number GSE44872.

Analysis of array data identified 358 genomic regions commonly enriched by the AS1 and T7 antibody datasets. The definitions of these genomic regional positions were based on TAIR7, which was also the basis for the GeneChip Arabidopsis Tiling 1.0R array design. We converted these TAIR7 positions into TAIR9-based ones by performing a matching analysis between probe sequences and TAIR9 genomic sequences. Consequently, we extracted 2135 genes located within 10 kbp from the enriched genomic regions by using positional relationship-based sequence analysis between genomic regions and genes (Takahashi et al., 2012a; Takahashi et al., 2012b).

For ChIP-PCR, chromatin samples were prepared from 21-day-old seedlings of as1-1 plants expressing pAS1:T7:AS1, as2-1 plants expressing pAS2:AS2:FLAG, and Col-0 plants expressing p3SS:GFP:AS1. Fragmented chromatin was immunoprecipitated with antibodies specific for T7, FLAG, GFP and AS1. Primers were designed to amplify regions of ~200 bp from 3 kb upstream to 1 kb downstream of ETT. Primer sequences used are listed in supplementary material Table S1.

Northern blotting and PCR
Quantitative real-time RT-PCR was performed as described by Matsumura et al. (Matsumura et al., 2009). Primer sequences used are listed in supplementary material Table S1.

Northern blotting of small RNAs was performed as described by Ueno et al. (Ueno et al., 2007). Locked nucleic acid-containing probe sequences are listed in supplementary material Table S1.

RESULTS
Direct targets of AS1 identified by ChIP-chip and expression array
To identify target genes of AS1-AS2, we searched for AS1-binding sites in the A. thaliana genome by ChIP-chip experiments (Fig. 1A). A DNA construct encoding the T7-tagged AS1 protein driven by its endogenous promoter (pAS1:T7:AS1) was introduced into the as1-1 mutant, and the resulting pAS1:T7:AS1 #5 line, which complemented the mutation (supplementary material Fig. S1A), was used for the ChIP-chip. Chromatin samples of 14-day-old seedlings were immunoprecipitated with antibodies specific for the T7 tag or the AS1 peptide. The resulting 358 genomic regions were identified (supplementary material Table S2). Detailed mapping of these sites in the A. thaliana genome revealed 2135 known or putative genes located within 10 kbp of the 358 AS1-binding sites (Fig. 1A; supplementary material Table S2).

In previous microarray and clustering analyses of gene expression profiles in shoot apices of 15-day-old plants of Col-0, as1-1, as2-1 and an ectopic overexpressor of AS2 cDNA (pAS1:AS2; designated AS2-eoe) in the as2-1 mutant, we showed that 382 genes were expressed in two clusters, 3 and 6: expression was enhanced in both
as1-1 and as2-1 plants, but suppressed in an ectopic overexpressor of AS2 (AS2-eoe) (Fig. 1B; supplementary material Fig. S1B) (Takahashi et al., 2008; Kojima et al., 2011; Takahashi et al., 2013). Cluster 3 included ETT and BP, the latter of which has been reported to be a direct target of AS1 (Guo et al., 2008), and cluster 6 included some of the abaxial determinant genes, such as KAN2.

By combining these 382 genes with those identified by the ChIP-chip experiments, we identified 35 genes in common (Fig. 1C; supplementary material Table S2). The selected 35 genes contained 12 transcription factors, including ETT and BP. Enrichment for these 12 transcription factors was significant (5.53-fold) (supplementary material Fig. S1C), and their transcript levels were increased in both as1 and as2 mutants and decreased or unchanged in lines with AS2 ectopic overexpression (AS2-eoe; Fig. 1D). As ETT is involved in leaf polarity regulation, we analyzed its regulation by AS1. As shown in Fig. 1E, a region 2.8 kb upstream of the translational initiation site of ETT was detected by ChIP-chip, suggesting that AS1 binds to this upstream region of ETT.

**AS1 binds the ETT promoter**

As shown in Fig. 1E, a region 2.8 kb upstream of the translational initiation site of ETT was detected by ChIP-chip. To confirm the binding of AS1 and/or AS2 to this region, we performed a ChIP-PCR assay with 34 primer pairs covering 3.5 kb upstream of the AS1-binding site, respectively, and introduced them into wild-type, as1, and as2 plants (Fig. 2A, B). Strong GUS activity was detected around the shoot apex, the hypocotyl and young growing leaves of the wild-type plants expressing the 4.9-kb reporter, but only weak or no GUS activity was detected in cotyledons or in mature first and second leaves (Fig. 2A). Strong and widespread GUS activity was detected, however, in mature leaves of as1-1 and as2-1.
as2-1 plants carrying the same construct (Fig. 2A). By contrast, the three genotypes showed GUS activity in mature leaves following transformation with the 2.3-kb construct that lacked the AS1-binding site (Fig. 2B). Furthermore, GUS expression in matured leaves of phenotypically wild-type plants with pETT4.9kb:GUS was comparable with plants carrying the same construct (Fig. 2C), whereas GUS expression in matured leaves of the wild type with pETT2.3kb:GUS was comparable with that of as1-1 in the same transgenic line (Fig. 2D). These data suggest that the region between the 2.3 kb and 4.9 kb upstream sites is involved in restriction of ETT expression in mature leaves, and that this repression is mediated by AS1 and AS2.

AS2 requires AS1 to regulate ETT expression directly
To examine whether transcript levels of the ETT gene are directly repressed by AS2, a line expressing AS2 fused to the glucocorticoid receptor (GR), under the control of the 35S promoter (35S:AS2:GR) (Ueno et al., 2007) was used. Transgenic plants were grown on MS plates and transferred to liquid MS medium with or without 10 µM dexamethasone (DEX) for various times (Fig. 2E). Analysis by real-time qRT-PCR showed that the level of ETT transcripts fell within 1 hour following DEX application. Transcript levels similarly fell when DEX was supplemented by 10 µM cycloheximide (CHX), an inhibitor of protein synthesis. By contrast, ETT transcript levels were not altered in DEX-treated as1-1 plants expressing AS2-GR (Fig. 2E).

ETT transcripts are negatively regulated by trans-acting siRNA-ARF (tasiR-ARF) (Allen et al., 2005; Williams et al., 2005), the biogenesis of which is mediated by the RDR6 gene. As shown in Fig. 2E, the reduction of ETT levels in AS2-GR plants supplemented with DEX was maintained in the rdr6 background, suggesting that the repression of ETT by AS2 does not require tasiRNA synthesis.

Unlike ETT, levels of its closely related ARF4, which is also a target of tasiR-ARF, did not fall within 4 hours after the addition of DEX or DEX plus CHX (Fig. 2E), nor did the levels of KAN2 and YAB5 transcripts (supplementary material Fig. S1F), which actually increased in as1 and as2 plants (Iwakawa et al., 2007).

These results suggest that AS2 represses the expression of ETT, but not that of ARF4, without de novo protein synthesis, independent of RDR6, but in the presence of wild-type AS1 function. This activity is likely to be mediated by AS1-AS2 bound to the 5’-upstream region of ETT and aided by the short half-life of ETT transcripts.

Indirect repression of ETT and ARF4 by AS1-AS2 is mediated by the miR390/RDR6 pathway
The transcriptional repression of ETT by AS1-AS2 is strong and rapid. However, ETT and its related ARF4 mRNAs are strongly regulated by tasiRNAs (Allen et al., 2005; Williams et al., 2005), and under such regulation, ubiquitous expression of ETT does not impact shoot development (Hunter et al., 2006). We therefore examined whether AS1 and AS2 might also be involved in regulation of ETT and ARF4 through the miR390 and tasiR-ARF pathway. We monitored the levels of miR390 and tasiR-ARF accumulation in DEX-induced 35S:AS2:GR plants (Fig. 3A). Accumulation of miR390 was first detected at 4 hours and then increased tenfold by 12 hours after treatment with DEX. This accumulation was abolished by the addition of CHX to the DEX treatment, suggesting that AS2 indirectly regulates the level of miR390. In contrast to miR390, however, no changes were detected in tasiR-ARF levels, even 12 hours after the DEX application. These results suggest that AS1-AS2 might regulate ETT expression at two levels: rapid regulation by direct repression, and slow regulation by indirect activation of miR390. Further characterization of the slow process was problematic, however, as incubation of 35S:AS2:GR plants with DEX greatly interfered with their growth. As an
alternative, 35S:AS2:GR plants were incubated on a solid medium containing 1 μM mifepristone (RU486; RU), which is a synthetic steroid compound (a weak agonist of DEX). Levels of miR390 gradually increased within 1-2 days of treatment with RU (Fig. 3B, lanes 2 and 3), whereas those of tasiR-ARF increased slightly by 3-4 days after RU treatment (Fig. 3B, lanes 4 and 5).

Because tasiR-ARF is generated by actions of several factors, including RDR6 (from the TAS3 transcript), we examined effects of a mutation in the RDR6 gene on accumulation of tasiR-ARF during AS2:GR induction. TasiR-ARF was not detected in rdr6-30 plants containing 35S:AS2:GR, even though the miR390 levels gradually increased by 1-2 days after RU treatment (Fig. 3C), suggesting that the tasiR-ARF accumulation by AS2:GR induction in wild-type plants is dependent on RDR6.

Levels of ETT transcripts decreased by 80-90% within 1 day of RU treatment in Col-0 (Fig. 3D). Levels of ETT transcripts also decreased by 50% within one day of RU treatment in the rdr6-30 background, but the reduction was less efficient than that in Col-0, and its levels gradually recovered (Fig. 3E). These results suggest that AS1-AS2 represses ETT transcript levels through the RDR6-dependent pathway in addition to the direct binding described above. ARF4 transcript levels were decreased twofold one day after RU treatment of Col-0 (Fig. 3F, lane 2). The amount of ARF4 transcripts did not decrease in rdr6-30 (Fig. 3G). These results suggested that AS1-AS2 represses ARF4 transcript levels only through the RDR6-dependent pathway.

**Loss of ETT and ARF4 rescues as1 and as2 mutant leaves**

The negative regulation of ARF3 and ARF4 by AS2, and the resemblance of plants overexpressing a tasiR-ARF-insensitive form of ETT to the as2 mutants (Hunter et al., 2006) suggested that part of the abnormal as1/as2 leaf morphology could be attributed to mis-expression of ARFs. To examine this assertion, the null alleles ett-13 and arf4-1 (Pekker et al., 2005) were crossed with as1 and as2 mutants.

As shown in Fig. 4A-C and supplementary material Fig. S2, several abnormalities of as2-1 plants were slightly suppressed by introduction of the ett-13 or arf4-1 single mutation into as2-1 plants. These include the formation of mediolateral asymmetric leaf lobes and leaflet-like structures, and downward curling of leaves. Neither the ett nor the arf4 mutation, however, restored the short leaf petioles of as1 or as2 mutant leaves (Fig. 4A). Introduction of ett-13 arf4-1 double mutations into as2-1 efficiently suppressed both downward curling of as2-1 cotyledons and asymmetric leaf organization (Fig. 4A-C). Similarly, the formation of leaf lobes and downward curling of as1-1 leaves were also abolished by the introduction of ett-13 arf4-1 (Fig. 4A). These results suggest that the several phenotypic abnormalities of as1 and as2 plants result from elevated expression of the ETT and ARF4 genes. Nevertheless, as we have shown before, the shorter as1 and as2 leaf petioles that were recovered by removal of class 1 KNOX genes BP, KNAT2 and KNAT6 activities (Ikezaki et al., 2010), remained unchanged by the introduction of either ett-13 or arf4-1 or both into as1 or as2 backgrounds (Fig. 4A). The wavy-surface phenotype of as2-1 leaves was partially rescued in as2-1 ett-13 arf4-1 (Fig. 4D).

When slices of as1 and as2 mutant leaves were incubated on MS medium without exogenous phytohormones, the frequency of shoot regeneration was higher, but that of root regeneration was lower than those found with wild-type leaf sections (Semiarti et al., 2001). Importantly, mutations in the class 1 KNOX genes (BP, KNAT2 and KNAT6), which are upregulated in as1-1 and as2-1, have not affected the increased shoot regeneration of as1-1 and as2-1 leaves (Ikezaki et al., 2010). By contrast, the frequency of shoot regeneration from leaf sections of the as1-1 ett-13 arf4-1 and as2-1 ett-13 arf4-1 triple mutants was restored to that of the wild-type sections (Fig. 4E,F). Our genetic analyses suggest, therefore, that AS1 and AS2 repress ARF4, in addition to ETT, and failure to restrict expression of both of them in as1 and as2 contributes to several aspects of their mutant leaves.

**Change of DNA methylation levels of the ETT gene in as1 and as2 plants**

As shown above (Fig. 3), fast transcriptional regulation and slower post-transcriptional regulation contribute to the AS1-AS2 repression of ETT. To examine whether lasting epigenetic modifications are also utilized, we searched public databases for hallmarks of epigenetic regulation in the ETT DNA. Cytosine residues at CG sites in exons 6 and 10 of the ETT gene in wild-type plants are strongly methylated, whereas cytosine residues were scarcely methylated at CG sites in other regions of the ETT locus including the 5'-upstream region (Zhang et al., 2006; Cokus et al., 2008).

We examined cytosine methylation of exons 6 and 10 of ETT in wild-type, as1-1, as2-1 and as2-eoe/as2-1 plants by bisulfite sequencing. As shown in Fig. 5A (positions a-f in exon 6), cytosine residues in CG pairs in exon 6 were completely methylated in the wild-type plants, validating the epigenomics database. Interestingly, CG methylation levels in exon 6 (positions c-f) were lower in both as1 and as2 mutants. As2-eoe/as2-1 had the same levels of CG methylation as the wild-type plants. We examined whether METHYLTRANSFERASE 1 (MET1), which is mainly responsible for maintaining CG methylation (Ronenus et al., 1996), is involved in regulation of ETT expression. Higher transcript levels of ETT were detected in shoot apices of the met1-1 mutant, but not in leaves (Fig. 5C). The levels of CG methylation in ETT were correlated with ETT expression levels.

We did not detect cytosine methylation around the AS1-binding region in any of the plant lines that we examined in this study. Levels of CG methylation in exon 10, which includes two tasiR-ARF recognition sites, varied among the wild-type, as1-1, as2-1 and AS2-eoe/as2-1 plants (supplementary material Fig. S3A, positions g-l). By contrast, CG methylation in exon 10 of ARF4, which also contains two tasiR-ARF recognition sites, was similar in the wild-type, as1-1, as2-1 and AS2-eoe/as2-1 plants (supplementary material Fig. S3B).

As the extent of CG methylation in exon 10 of either ETT or ARF4 was not correlated with the mutant phenotype, we speculate that although as1 and as2 mutations affected the CG methylation status in these sites, these events might not be related to the downregulation of ETT and ARF4 by tasiR-ARF.

To examine the effects of DNA methylation of the ARF genes on leaf structure, we treated wild-type, as1 and as2 plants with 5-aza-2'-deoxycytidine, which inhibits methylation of DNA. As shown in Fig. 5D, filamentous leaves were generated in as1-1 and as2-1 plants but not in wild type, suggesting that the inhibitor induced defects in adaxial-abaxial polarity establishment in the mutant plants. Such synergistic effects are typical of many other mutations that enhance adaxial-abaxial polarity establishment in the mutant plants. Our genetic analyses suggest, therefore, that AS1 and AS2 repress ARF4, in addition to ETT, and failure to restrict expression of both of them in as1 and as2 contributes to several aspects of their mutant leaves.
cytosine residues in CG pairs in exon 6 of wild-type plants treated with 5-aza-2'-deoxycytidine, whereas levels of CG methylation in exon 6 were lower in both as1 and as2 plants treated with 5-aza-2'-deoxycytidine than those levels in both untreated and treated wild type. Furthermore, the extent of CG methylation in exon 6 was correlated with phenotypic severity.

These results suggest that the leaf patterning abnormalities of as1 and as2 plants treated with the inhibitor of DNA methylation primarily resulted from elevated expression of ETT at the shoot apex. In addition, these results revealed that AS1 and AS2 mediated DNA methylation independently of MET1.

**DISCUSSION**

In the present study, we showed that AS1-AS2 represses ETT directly, then represses both ETT and ARF4 indirectly through the RDR6-dependent pathway, and regulates leaf development in the adaxial-abaxial and medial-lateral directions, resulting in a flat and symmetrical leaf lamina (Fig. 6A). In addition, DNA methylation of ETT, in which AS1-AS2 is involved, might be related to establishment of the adaxial-abaxial leaf polarity (Fig. 6A,B). AS1-AS2 might regulate ETT and ARF4 temporally by dual regulation in early stages of the leaf developmental process (Fig. 6C).

**Suppression of ARF3/4 activities by AS1-AS2 stabilizes the adaxial-abaxial partitioning of leaves**

Many genes are involved in regulation of adaxial-abaxial partitioning of *A. thaliana* leaves (Fig. 6C). Products of the first gene class include the class III HD-ZIP proteins, which specify
the adaxial leaf domain; in their absence, radial and abaxialized organs are formed (Alvarez et al., 2006; Bowman and Floyd, 2008). The KANADI genes specify the abaxial side, and in their absence, too, leaves are nearly radial (Eshed and Bowman, 2004; Bowman and Floyd, 2008). The second class also includes genes involved in this process; however, in their absence, flat abnormal bifacial leaves develop. This class includes the AS1 and AS2 genes, which promote adaxial leaf identity (Byrne et al., 2000; Iwakawa et al., 2002; Iwakawa et al., 2007) and the ARF genes ETT and ARF4, which promote abaxial identity (Pekker et al., 2005). The third group includes the abaxially expressed YAB/FIL genes, which direct lamina formation and translate the polarity into growth (Sarojam et al., 2010). The fourth, and by far the largest, group includes many genes without indicative mutant phenotypes, which in specific genetic combinations can transform one cell type into another. We consider members of the first group as the patterning initiators (Bowman and Floyd, 2008; Efroni et al., 2010); the second group as stabilizers (Pekker et al., 2005) (this study); the third group as translators (Sarojam et al., 2010); and the fourth group as modifiers of adaxial-abaxial patterning (Szakonyi et al., 2010).

There are complex regulatory relationships among members of the different groups. For example, YABBY activity is required to maintain expression of the class III HD-ZIP genes, whereas KAN1 acts to restrict AS2 expression to the adaxial leaf domain. The expression of YABBY genes is dependent upon earlier KAN activity.
The mutual regulations among these genes also contribute to the establishment of adaxial-abaxial leaf polarity in *A. thaliana* (Fig. 6C; Sarojam et al., 2010; Kelley et al., 2012). Notably, all members of initiators and stabilizers are transcriptional regulators. All are expressed in a polar manner with two exceptions; *AS1* and *ETT* are expressed throughout early stages of leaf primordia. Although these two factors have overlapping expression, we showed here that *AS1-AS2* acts to restrict the expression for establishment of leaf adaxial-abaxial polarity. The leaf primordia are contained entirely within the shoot apical meristem (SAM), and then it begins to grow outwards. The primordium has acquired polarity in the radial dimension. At early stages, outgrowth from the SAM is conspicuous (Lynn et al., 1999). Black lines indicate direct regulation and dashed black lines indicate indirect regulation.

The mutual regulations among these genes also contribute to the establishment of adaxial-abaxial leaf polarity in *A. thaliana* (Fig. 6C; Sarojam et al., 2010; Kelley et al., 2012).

Notably, all members of initiators and stabilizers are transcriptional regulators. All are expressed in a polar manner with two exceptions; *AS1* and *ETT* are expressed throughout early stages of leaf primordia. Although these two factors have overlapping expression, we showed here that *AS1-AS2* acts to restrict *ETT* at several levels: temporally, transcriptionally, post-transcriptionally and via epigenetic modifications of its DNA (Fig. 6B). Moreover, several abnormalities of *as1* or *as2* leaves are restored when the activities of *ETT*, *ARF4* or both, are eliminated. Thus, our work provides a mechanistic basis for the ‘stabilizing’ role of *AS1-AS2* via fine-tuning of *ETT* and *ARF4* levels, activities of which modulate the initiator KAN proteins for adaxial-abaxial leaf patterning. We propose that genes of the second group, *AS1*, *AS2*, *ETT* and *ARF4*, be termed ‘stabilizers’.

**Direct repression of *ETT* by *AS1* and *AS2**

Our observations indicated that *AS1* binds to the 5’-upstream region of *ETT*, whereas *AS2* appeared to bind only very weakly to this region. This result might be attributable to differences in affinity for the *AS1*-binding region. It is possible that *AS2* might not bind to DNA directly, but instead binds indirectly via its interaction with *AS1* or some unknown factors. Interactions between *AS1* and *AS2* have been detected in vitro (Phelps-Durr et al., 2005; Guo et al., 2008; Yang et al., 2008; Szakonyi et al., 2010), but such interactions remain to be confirmed in vivo. The molecular role of *AS2* in interactions with *AS1* bound to the 5’-upstream region of *ETT* in vivo remains to be clarified.

We investigated whether DNA regions 3, 4 and 5 in the *ETT* locus (Fig. 1E) might contain obvious motifs identified in *AS1* complex-binding sites at BP (Guo et al., 2008). Guo et al. (Guo et al., 2008) proposed that *AS1* binds the 5’-upstream regions of BP, and these regions include two motifs: motif I (CWGTTD), which is a c-Myb-related sequence (CNGTTD), and motif II (KMKTTGAHW). Our sequence analysis revealed that three c-Myb-related sequences (~2736 to ~2731, ~2782 to ~2777, and ~2882 to ~2877) and one motif II-related sequence (~2752 to ~2744) were present in regions 3 and 4 of *ETT*. Additionally, we found a GTT repeated sequence in region 4 of *ETT*, which is also found in Myb-SANT-like transcription factor-binding sequences (England et al., 1990; Lang and Juan, 2010). These sequences found in the present study should be tested further for the binding of *AS1-AS2* to repress *ETT*.

**Repression of both *ETT* and *ARF4* by *AS1* and *AS2* via the miR390/RDR6 pathway**

The present results have shown that *ETT* and *ARF4* expression is also controlled through pathways via *AS1-AS2*-mediated and RDR6-dependent post-transcriptional gene silencing (PTGS) (Fig. 3B-G; Fig. 6). Despite the clear involvement of RDR6 in the *AS1-AS2*-mediated PTGS of *ETT* and *ARF4* expression, the role of *tasiR-ARF* in the PTGS might still be ambiguous, however, because the increase in *tasiR-ARF* accumulation was observed after the *ETT* and *ARF4* mRNAs levels decreased (Fig. 3). Two explanations could be possible: (1) although sufficient *tasiR-ARF* for downregulation might be induced by *AS1-AS2*, the level might be undetectable; (2) another unidentified *tasiR-ARF* might be generated by *AS1-AS2* and the miR390/RDR6-dependent pathway at an earlier stage of *ETT* and *ARF4* downregulation.

Despite these possibilities, control of the ARF genes by *AS1-AS2* through the RDR6-dependent pathway, probably including *tasiR-ARF*, might be significant for the following reasons. Our observations are consistent with the previous report that overexpression of *ETT*, which is a nondegradable mutant of *ETT* mediated by *tasiR-ARF*, yields a phenotype similar to that of *as2* (Hunter et al., 2006). Mutations of factors involved in the biogenesis of *tasiR-ARF* enhance the phenotypes of *as1* and *as2* mutations, generating filamentous leaves (Kidner and Martienssen, 2005; Li et al., 2005; Garcia et al., 2006; Yang et al., 2006; Xu et al., 2006; Kojima et al., 2011). The phenotypic enhancement by mutations of genes for the biogenesis of *tasiR-ARF* implies that this pathway might be also modulated by an unidentified factor.
Epigenetic regulation of ETT by AS1-AS2 for establishing the adaxial leaf domain

We showed that levels of DNA methylation in exon 6 of ETT were depressed in both as1 and as2 mutants. It was reported that over one-third of expressed genes in A. thaliana contain DNA methylation within their transcribed regions (Zhang et al., 2006; Vaughan et al., 2007; Zilberman et al., 2007; Cokus et al., 2008; Lister et al., 2008), and loss of methylation in the transcribed regions of these genes results in enhanced levels of transcription (Zilberman et al., 2007). Recently, it has been verified that DNA demethylation increases ETT expression in a mutant for MET1 (Li et al., 2011). We also observed increased levels of ETT transcripts in shoot apices of met1 (Fig. 5C). As the promoter of ETT is not methylated, gene body methylation would be involved in transcriptional regulation of ETT, and the decreased level of gene body methylation might increase the transcription level of ETT in as1 and as2, as observed in met1. It would be interesting to elucidate how ASI and AS2 mediate DNA methylation of ETT by additional pathways parallel with regulation by MET1.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.orglookup/suppl?doi=10.1242dev.085365-S+RCD1

References


**ARF3** is a direct target of AS1


**Fig. S1. Clustering analysis of expression array and ChIP-PCR assay in the ARF3 locus.** (A) Typical examples of the transgenic plants used for ChIP-chip and ChIP-PCR assays. The presence of pAS1:T7:AS1 complemented the as1-1 mutant phenotype, and the presence of pAS2:AS2:FLAG complemented the as2-1 mutant phenotype. Schematics of the construction of pAS1:T7:AS1 and pAS2:AS2:FLAG are shown. The green and blue boxes indicate T7-tag and FLAG-tag, respectively. Black and white boxes indicate coding and non-coding exons, respectively. G. (B) Clustering analysis by KB-FuzzyART. Levels of gene expression in comparison with those of Col-0 (WT) are shown. Clusters 3 and 6 contained genes having elevated transcription levels in as1-1 and as2-1. (C) Enrichment of genes for transcription factors among 35 selected genes, and heatmap and hierarchical clustering. The ratio of genes for transcription factors to all genes in *Arabidopsis* is 6.20% (2061/33,239) in the TAIR9 database (http://www.arabidopsis.org/). The ratio was 34.3% (12/35) in the case of the 35 selected genes. Therefore, the enrichment rate was estimated as 5.53 (P=6.83×10⁻⁷, as calculated by Fisher’s exact test). For the signal log2 ratio of 12 transcription factors, hierarchical clustering was performed by using Euclidean distance and average linkage. In the present study, we merged three databases for transcription factors: namely, the *Arabidopsis* transcription factor database (AtTFDB; http://arabidopsis.med.ohio-state.edu/AtTFDB/), the *Arabidopsis thaliana* Regulatory Network (AtRegNet; http://arabidopsis.med.ohio-state.edu/REIN/), and the transcription factors portion of the Gene Ontology database (http://www.geneontology.org/). In all, 2374 transcription factors were identified as the products of independent genes. (D) Schematic of the ARF3 genomic region. Boxes indicate exons. Relative positions of primer pairs used in ChIP-PCR assays are shown. (E) Results of ChIP-PCR assays of plants harboring p35S:AS1:GFP with GFP- and AS1- specific antibodies, as indicated. (F) Expression analysis of *BP*, *KAN2* and *YAB5* genes in 35S:AS2:GR plants. Relative expression levels of the *BP*, *KAN2* and *YAB5* genes in 7-day-old 35S:AS2:GR plants after treatment with DEX (gray bars) or DEX and CHX (white bars) for the times indicated.
Fig. S2. Mutations in *ARF3* and *ARF4* suppressed major phenotypes of *as1* and *as2*. Representative gross morphology of 40-day-old plants and their leaves are shown. The genotype of each plant is indicated. Scale bars: 5 mm.
Fig. S3. Effect of 5-aza-2′-deoxycytidine on leaf polarity and levels of cytosine methylation of the ARF3 and ARF4 loci in Col-0, as2-1, AS2-ee/AS2-1 and as1-1 plants. (A) Schematic of the ARF3 locus. Gray boxes indicate exons. The region delineated by a dashed line (exon 10) was examined for cytosine methylation. Vertical bars indicate percentages of methylated cytosines in CG pairs. Red and blue bars indicate cytosines with decreased and increased levels of methylation, respectively, in the as1 and as2 mutants. Green bars indicate a decreased level in the as1 mutant and an increased level of methylation in the as2 mutant. (B) Schematic of the ARF4 gene. Gray boxes indicate exons. Exon 10 was examined for cytosine methylation. Vertical bars indicate percentages of methylated cytosines in CG pairs. The tasiR-ARF recognition sites are indicated as purple lines.
Table S1. Sequences of primers used for real-time RT-PCR, ChIP-PCR, Northern blotting of small RNA and bisulfite sequencing

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Table S2 Summary of ChIP-chip and expression array analysis  

Download Table S2