Development 140, 1958-1969 (2013) doi:10.1242/dev.085365


# 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).

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 (Waites and Hudson, 1995; Waites 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; Sawa 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 Zambryski, 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; Gasciolli et al., 2005; Xie et al., 2005; Adenot et al., 2006; Montgomery et al., 2008).

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**Accepted 18 February 2013**

The *asymmetric leaves1* (*as1*) mutant is disrupted in the *PHAN MYB* ortholog. AS1 forms a complex with ASYMMETERIC 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 lobes 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 primordia (Byrne et al., 2000; Iwakawa et al., 2002; Iwakawa et al., 2007; Keta et al., 2012). AS2 proteins are localized to subnuclear bodies adjacent to the nucleoli in leaf cells, called AS2 bodies, and are also dispersed in the nucleoplasm; AS1 proteins are located as speckles in the nucleoplasm and are also concentrated in the AS2 bodies by an AS2-dependent process (Ueno et al., 2007; Luo et al., 2012). AS1 and AS2 form the AS1-AS2 complex (Guo et al., 2008; Yang et al., 2008), which represses the expression of two class 1 KNOX genes, *BP* and *KNAT2*, by binding to their respective promoter regions (Guo et al., 2008), showing that these KNOX genes are direct targets of AS1-AS2. In addition, the *AS1* and *AS2* genes repress the expression of genes for abaxial determinants, such as *KAN2*, *ETT* and *YAB5* (Iwakawa et al., 2007; Takahashi et al., 2008). The presence of other unidentified direct targets, however, has also been proposed (Ikezaki et al., 2010). Although epigenetic repression of KNOX genes by AS1 and AS2 has been hypothesized (Phelps-Durr et al., 2005), mechanisms involved in epigenetic regulation during leaf development are largely unknown.

Here, we characterized the direct target genes of the AS1-AS2 complex, showing that it regulates *ETT* transcription directly and, furthermore, indirectly via the miR390- and RDR6-dependent pathway. Additionally, *AS1* and *AS2* maintain the status of gene body methylation of the *ETT* gene. The repression of *ETT* by AS1-AS2 is important for establishment of adaxial-abaxial and medial-lateral polarity of leaves in *A. thaliana*, and we suggest that AS1-AS2 functions to stabilize previously determined leaf patterning mechanisms.

## MATERIALS AND METHODS

### Plants and plasmid construction

Details of Col-0 (CS1092), *as1-1* (CS3374), *as2-1* (CS3117) and *rdr6-30* 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 dexamethasone (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 pETTΔMAR:GUS plasmid (herein after designated as pETT4.9kb:GUS) (Ng et al., 2009) was digested with *Not*I and *Nde*I, filled with the Klenow fragment of DNA polymerase, and self-ligated to generate pETT2.3kb:GUS. Schematic representation of pAS1:T7: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 T7F1SalMun (5′-CTCAATTGTCGACT-ATGGCTAGCATACTGTG-3′) and AS1R1NotI (5′-GAGCGGCCGC-TCAGGGGCGGTCTAATCTGCAAC-3′) with pBSSK:AS1 (Iwakawa et al., 2002) as a template. We then inserted the amplified sequence between the *Eco*RI and *Sph*I sites of pBSSK:AS1. The resultant fusion construct was cloned into the pGreen0029 binary vector (Hellens et al., 2000) to yield pAS1:T7:AS1. The coding region of AS2 from which the termination codon had been deleted was fused to the sequence for three tandem FLAG tags that was amplified by PCR with primers pU330-FLAGS 5′-CTAGGGACTACAAGGATCACGATGGGGATTATAAAGACCACGA-CATTGACTACAAGGATGACGATGACAAGCCCGGG-3′ and pU331-FLAGA (5′-GATCCCCGGGCTTGTCATCGTCATCCTTGTAGTCA-ATGTCGTGGTCTTTATAATCCCCATCGTGATCCTTGTAGTCC-3′). After digestion with *Bgl*II, the fusion construct was isolated and inserted between the *Bgl*II and *Not*I sites of pBS35SAS2YFP (Ueno et al., 2007). The resultant plasmid was designated pAS2:3xFLAG. The fragment containing the AS2:FLAG gene was amplified from pAS2:3xFLAG by PCR with primers AS2F1Sal (5′-GAGTCGACATGGCATCTTCTTC-AACAAACTCAC-3′) and FLAGR2NotXho (5′-AACTCGAGCGGC-CGCTCACTTGTCATCGTCATCC-3′). After digestion with *Xho*I, the fragment was inserted between the *Aar*I and *Sal*I sites of pAS2-TAS2, which was constructed by the insertion of the 5′ region of *AS2* (nucleotides –3301 to –1), the recognition sites of *Aar*I and *Sal*I, and the 3′ region of *AS2* (nucleotides +595 to +2868), into the *Apa*I/*Eco*RV sites of pGreen0029 (Hellens et al., 2000). The resulting plasmid was designated pAS2:AS2:FLAG. 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 357 to 373 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:T7: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 0.7-μm resolution 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 Affimetrix GeneChip Command Console. Raw CEL data files obtained from tiling array experiments were analyzed with Tiling Analysis Software

(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 p35S: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. Bisulfite conversion of genomic DNA extracted from seedlings was as described by Kaneda et al. (Kaneda et al., 2004). PCR was performed with primers listed in supplementary material Table S1. Amplified fragments were cloned into the pGEM-T Easy Vector (Promega, Tokyo, Japan) and sequenced.

## 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

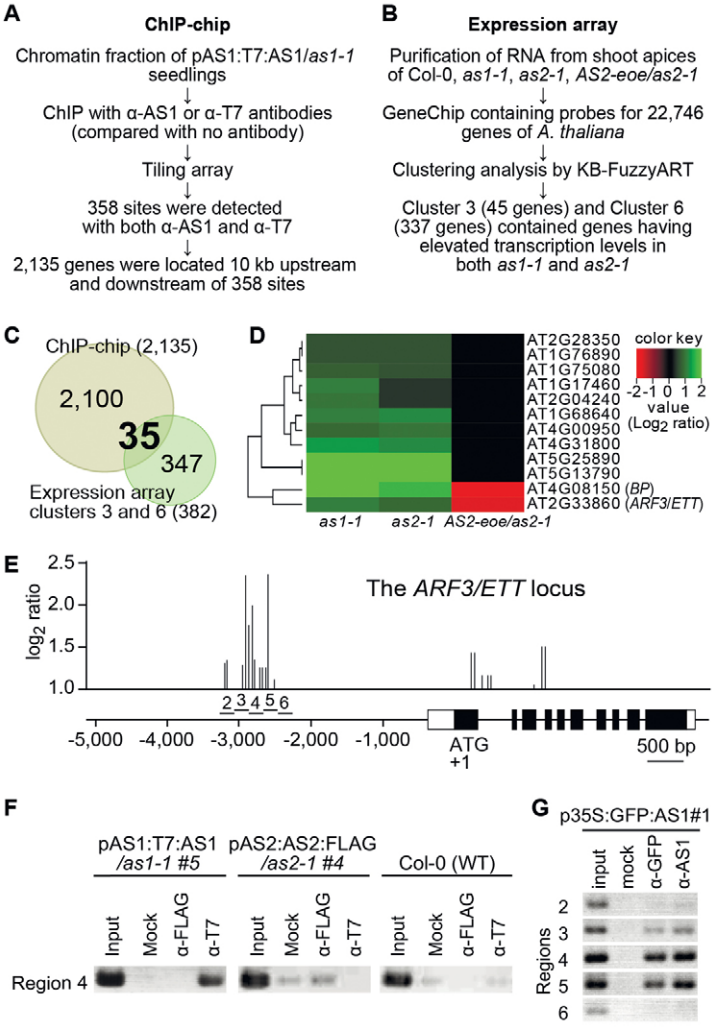


**Fig. 1. ChIP-chip analysis, expression array and ChIP-PCR assay reveal that *ETT* is the direct target of *AS1*.** (**A**) Experimental layout to monitor ChIP-chip analysis. (**B**) Experimental layout to monitor the expression array and clustering analysis by KB-FuzzyART. Array data have been deposited in the NCBI Gene Expression Omnibus under accession number GSE44028 (Takahashi et al., 2008; Takahashi et al., 2013). (**C**) Venn diagram of genes overlapping the AS1-binding genes resulting from ChIP-chip analysis and AS1-regulated genes identified by gene expression profiling. Clusters 3 and 6 contain genes negatively regulated by *AS1* and *AS2*. (**D**) Heatmap representing transcript levels of 12 transcription factors among the 35 genes that are candidates for being direct targets of AS1. (**E**) Binding profile of AS1 to the upstream regulatory region of *ETT*, as determined by ChIP-chip. $Log_2$ ratios of signal intensities (*y*-axis) are shown at genomic positions indicated in the schematic diagram of the *ETT* genomic region. Black and white boxes indicate coding and non-coding exons, respectively. Relative genomic positions from the translational start site (ATG) are indicated. Positions of primer pairs used in the ChIP-PCR assays are shown. (**F**) ChIP-PCR assay performed on pAS1:T7:AS1*/as1-1*, pAS2:AS2:FLAG*/as2-1* and Col-0 (WT) plants. Chromatin samples immunoprecipitated with T7- or FLAG-specific antibodies were analyzed by PCR. Total input chromatin extract and mock-precipitated extracts were used as controls. (**G**) ChIP-PCR assay performed on p35S:GFP:AS1/Col-0 plants. Chromatin samples immunoprecipitated with GFP- or AS1- specific antibodies were analyzed by PCR.

*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 *ETT* coding sequence to 0.2 kb downstream of the translation termination codon (supplementary material Fig. S1D). Of the 34 DNA regions, one [#4, −2801 to −2601 upstream of the *ETT* translation start (Fig. 1E)] was reproducibly amplified from chromatin isolated from the pAS1:T7:AS1 #5 line and immunoprecipitated with antibodies specific for T7 (Fig. 1E,F). Conversely, this region was not enriched in mock-treated chromatin from the same line or chromatin prepared from wild-type plants (Fig. 1F).

We next prepared chromatin samples from the p35S:GFP:AS1 #1 line and performed immunoprecipitation with antibodies specific for GFP or AS1. Genomic regions 3, 4 and 5 were thus amplified from the immunocomplexes (Fig. 1G; supplementary material Fig. S1E). ChIP-PCR performed with chromatin samples of pAS2:AS2:FLAG plants (supplementary material Fig. S1A) and immunoprecipitated with antibodies against FLAG amplified only region #4, albeit at a weaker level (Fig. 1F).

## An upstream region of *ETT* directs transcriptional repression by AS1 and AS2

We generated a pair of GUS reporter constructs including either a 4.9- or 2.3-kb region upstream of *ETT*, which contained or lacked 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

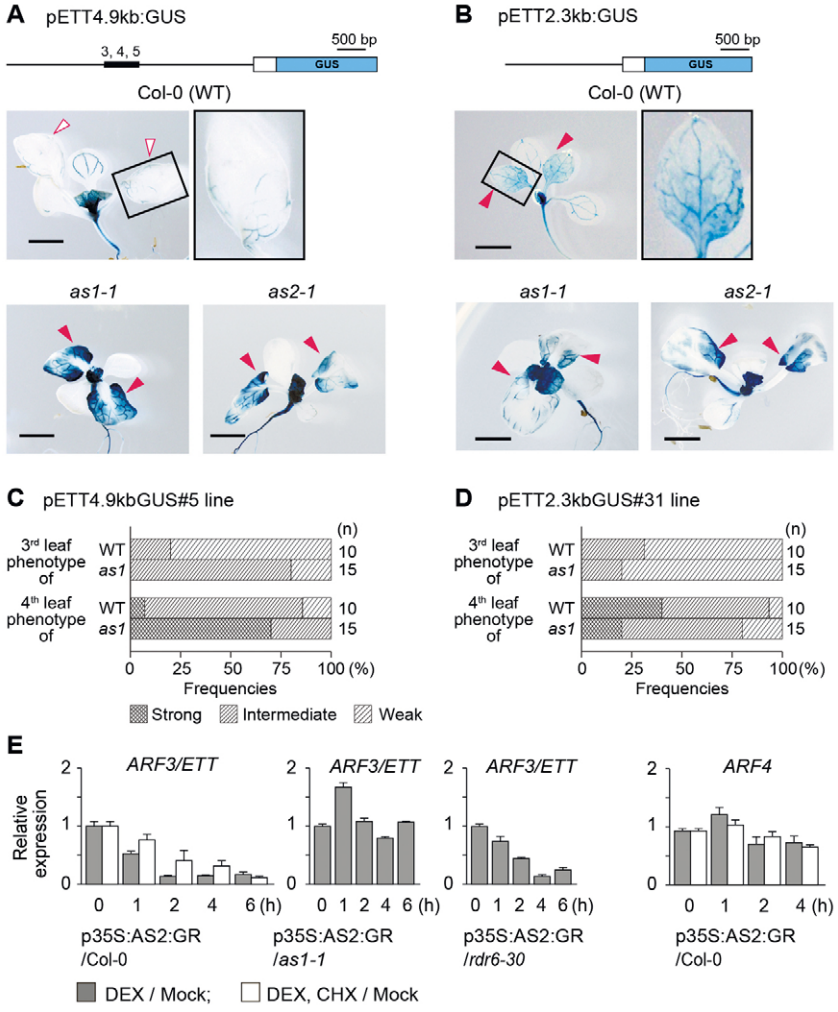


**Fig. 2. Analysis of regulation of *ETT* expression by *AS1* and/or *AS2*.** (**A**) Schematic of the 4.9-kb GUS construct (pETT4.9kb:GUS), including AS1-binding regions 3, 4 and 5 in *ETT*, indicated by the black box. The blue box indicates the GUS gene. Representative GUS expression patterns of Col-0 (WT), and *as1-1* and *as2-1* plants (18-day-old T1) carrying the pETT4.9kb:GUS construct are shown. Red arrowheads indicate GUS signals in first and second leaves; white arrowheads surrounded by red lines indicate first and second leaves, in which no GUS signal was detected. Magnified view of the leaves in Col-0 is shown. (**B**) Schematic of the 2.3-kb GUS construct (pETT2.3kb:GUS), which does not include the AS1-binding regions 3, 4 and 5. The blue box indicates the GUS gene. Representative GUS expression patterns of Col-0 (WT), and *as1-1* and *as2-1* plants (18-day-old T1) carrying the pETT2.3kb:GUS construct are shown. Red arrowheads indicate GUS signals in first and second leaves. Magnified view of the leaves in Col-0 is shown. (**C**,**D**) Frequencies of plants categorized by staining strength of GUS in third and fourth leaves in of the pETT4.9kb:GUS#5 line (C) and the pETT2.3kb:GUS#31 line (D). (**E**) Expression analysis of the *ARF* genes in Col-0 (WT), *as1-1* and *rdr6-30* backgrounds. Relative expression levels of *ETT* and *ARF4* in 7-day-old 35S:AS2:GR plants after treatment with DEX (gray bars) or DEX and CHX (white bars) for the times (h; hours) indicated. Values were normalized to the respective mock-treated controls. *ETT* transcript levels in 35S:AS2:GR/Col-0, 35S:AS2:GR/*as1-1* and 35S:AS2:GR/*rdr6-30* plants were measured by real-time qRT-PCR after treatment with DEX for the times indicated. Values were normalized to the levels of *ACTIN 2* transcripts and further normalized to the respective mock-treated controls. Error bars represent s.d. Scale bars: 5 mm.

*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 weaker compared with that of *as1-1* phenotype plants in the same transgenic line (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 ta-siRNA 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 ta-siRNAs (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

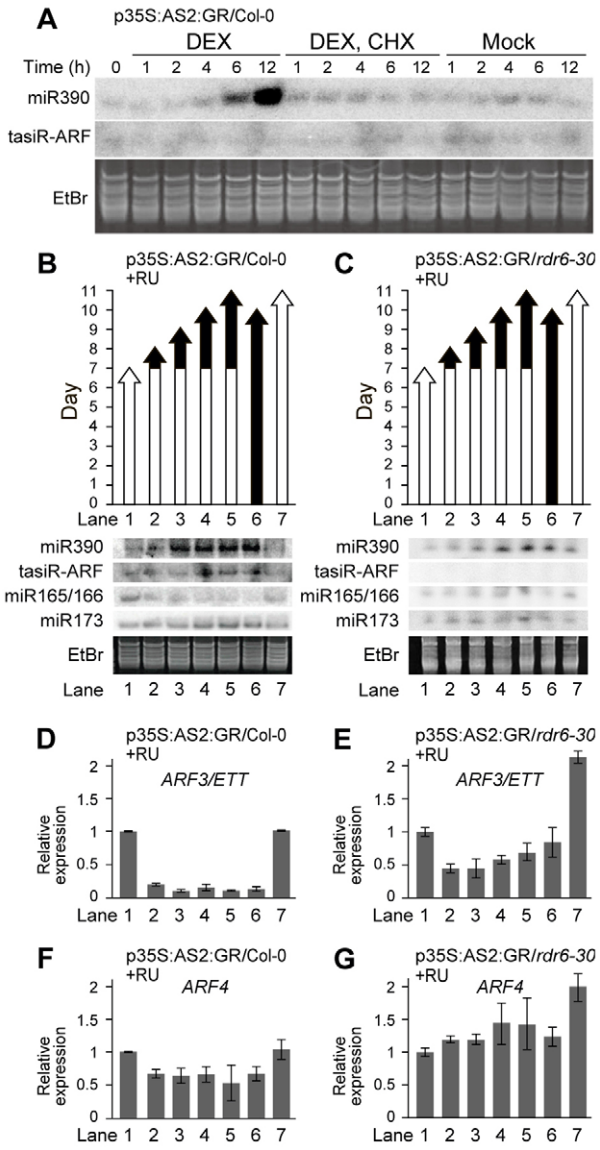


**Fig. 3. *AS1* and *AS2* regulate the miR390- and RDR6-dependent pathway.** (**A**) Northern analysis of small RNAs in 7-day-old plants with 35S:AS2:GR after treatment with DEX or DEX and CHX for the times indicated. (**B**,**C**) 35S:AS2:GR/Col-0 (B) and 35S:AS2:GR/rdr6-30 (C) plants were grown for 7 days on solid medium without RU and then transferred onto medium containing 1 μM RU for 1 to 4 days (lanes 2 to 5). As positive and negative controls, plants were grown for 10 days on medium with 1 μM RU (lane 6), and for 7 and 11 days on medium without RU (lanes 1 and 7), respectively. Accumulated levels of small RNAs at each lane are shown. (**D-G**) Relative expression levels of *ETT* (D,E) and *ARF4* (F,G) in 35S:AS2:GR/Col-0 (D,F) and 35S:AS2:GR/rdr6-30 (E,G) plants as measured by real-time qRT-PCR after the RU treatment for the indicated lanes as described for panels B,C. Values were normalized to the respective controls in lane 1. Expression values were normalized to the *ACTIN 2* transcript levels. Error bars represent s.d. EtBr, ethidium bromide.

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 *ARF*s. 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 did the wild-type plants. We examined whether *METHYL TRANSFERASE 1* (*MET1*), which is mainly responsible for maintaining CG methylation (Ronemus 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 the mutant phenotype of *as1* or *as2*. As shown in Fig. 5D,E, the filamentous leaf phenotype of 5-aza-2′-deoxycytidine-treated *as1* and *as2* plants was partially suppressed by introduction of the *ett-13* mutation into these plants, and was completely suppressed by the introduction of *ett-13 arf4-1* double mutations.

We next examined cytosine methylation of *ETT* exon 6 in wild-type, *as1-1* and *as2-1* plants treated with 5-aza-2′-deoxycytidine (Fig. 5B). Compared with untreated plants, there were slightly fewer

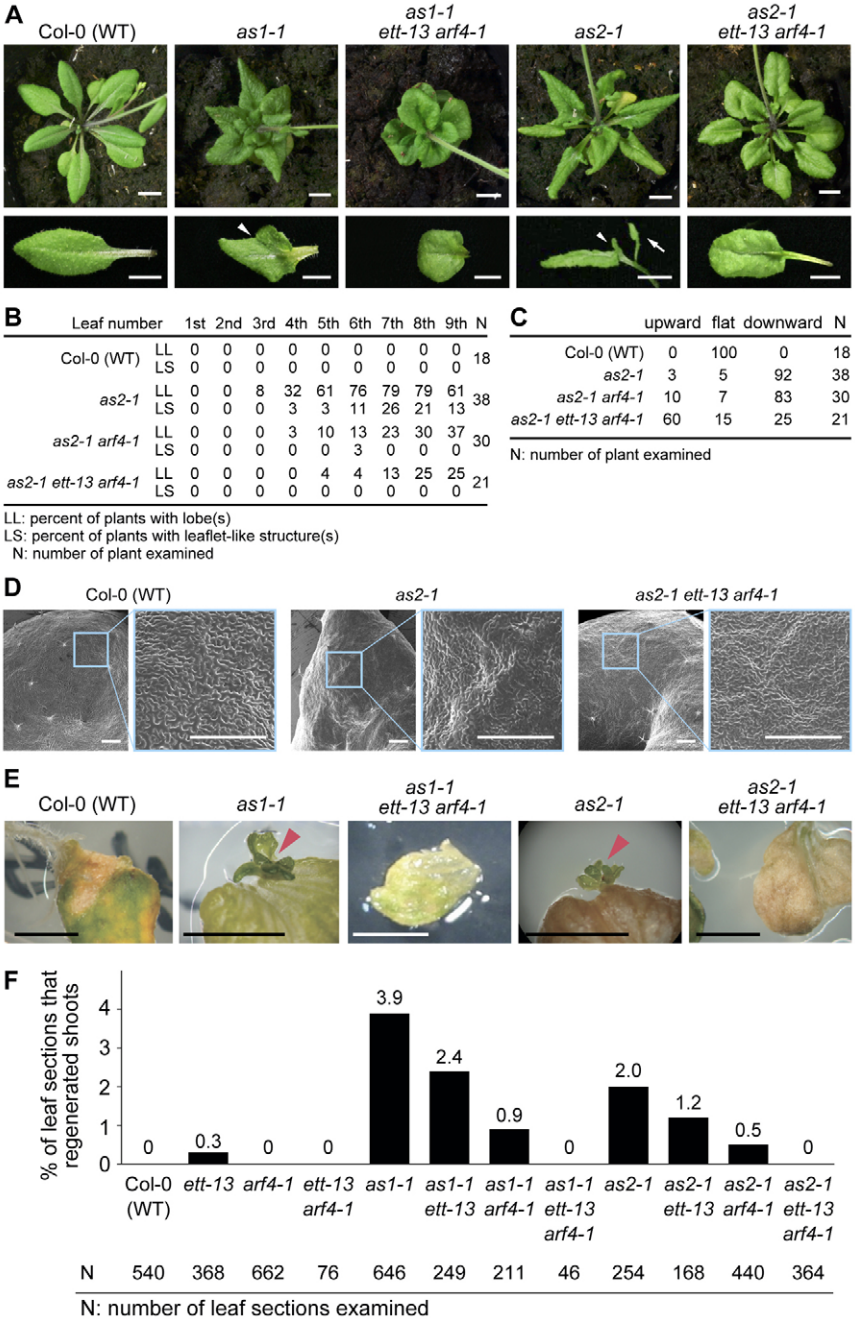


**Fig. 4. Mutations in *ETT* and *ARF4* suppressed major phenotypes of *as1* and *as2*.**
(**A**) Representative gross morphology of 40-day-old plants and magnified views of their leaves. The genotype of each plant is indicated. Arrowheads indicate leaf lobes and arrow indicates a leaflet-like structure. Scale bars: 5 mm (upper) and 2 mm (lower). (**B**) Positions and percentages of leaves with lobes and leaflet-like structures in 40-day-old Col-0 (WT), *as2-1*, *as2-1 arf4-1* and *as2-1 ett-13 arf4-1* plants. (**C**) Percentages of 9-day-old plants with upwardly curled, flat or downwardly curled cotyledons. (**D**) Scanning electron micrographs of the adaxial side of the first leaves of 20-day-old plants of Col-0, *as2-1* and *as2-1 ett-13 arf4-1* grown on soil. Scale bars: 500 μm. (**E**) Adventitious shoots were regenerated from *as1-1* and *as2-1*, but were rarely observed for Col-0 (WT), *as1-1 ett-13 arf4-1* or *as2-1 ett-13 arf4-1*. Red arrowheads indicate shoots generated from leaf sections. Scale bars: 5 mm. (**F**) Regeneration frequencies of adventitious shoots from leaf sections incubated for 21-24 days on MS medium. The numbers of leaf sections examined are shown below the graph.

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 adaxal-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

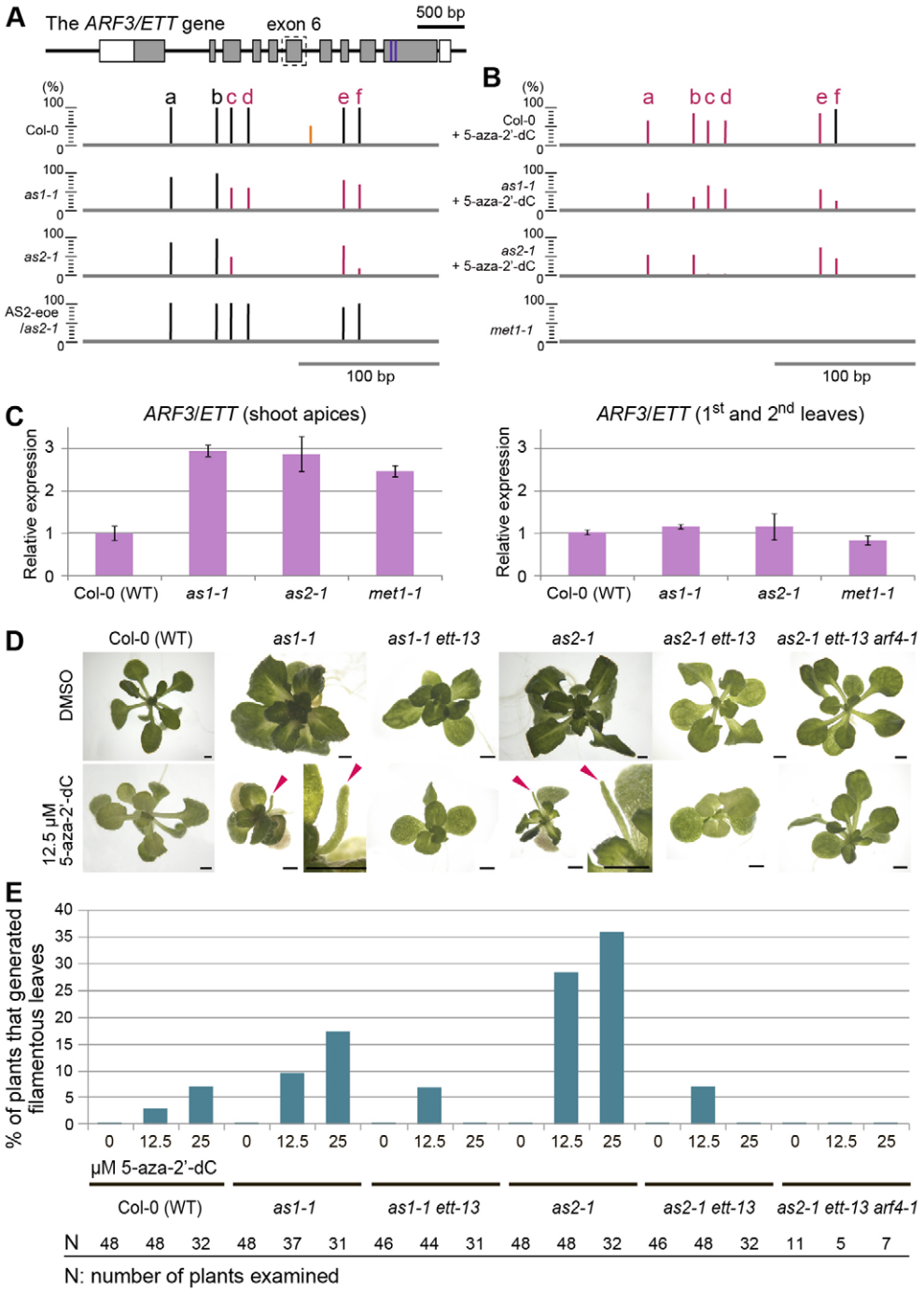


**Fig. 5. AS1 and AS2 maintain DNA methylation in *ETT* to produce flat leaves.** (**A**,**B**) Levels of cytosine methylation in the region of exon 6 of *ETT*. Schematic of the *ETT* locus is shown. Gray boxes indicate exons. The region outlined by a dashed line (exon 6) was examined for cytosine methylation in above-ground parts of 15-day-old plants. Ten clones were sequenced. Vertical bars indicate the percentage of methylated cytosines in CG and CHG pairs. Red bars indicate cytosines in CG with decreased methylation levels compared with those in Col-0. The methylation level and position of CHG is indicated by an orange bar. (A) Levels of cytosine methylation in Col-0, *as1-1*, *as2-1* and *AS2-eoe/as2-1* were examined. (B) Levels of cytosine methylation in Col-0, *as1-1* and *as2-1* treated with 12.5 µM 5-aza-2′-deoxycytidine (5-aza-2′-dC), and the *met1-1* mutant were examined. (**C**) Relative transcript levels of *ETT* in Col-0 (WT), *as1-1*, *as2-1* and *met1-1* were quantified by real-time qRT-PCR. RNA was isolated from the shoot apices (left) or first and second leaves (right) of 15-day-old plants. Each value was normalized to the level of *ACTIN 2* transcripts. Values are shown relative to the value for wild-type plants. Error bars represent s.d. ($n$=3). (**D**) The plants were grown for 21 days on MS medium containing DMSO vehicle and 12.5 µM 5-aza-2′-deoxycytidine in DMSO. Red arrowheads indicate filamentous leaves. The filamentous leaves in *as1-1* and *as2-1* treated with 5-aza-2′-deoxycytidine were restored to flat leaves by the *ett* and *arf4* mutations. Scale bars: 1 mm. (**E**) Frequencies of plants with filamentous leaves in *as1-1*, *as1-1 ett-13*, *as2-1*, *as2-1 ett-13* and *as2-1 ett-13 arf4-1* mutants treated with 5-aza-2′-deoxycytidine. The numbers of plants examined are shown below the graph.

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.

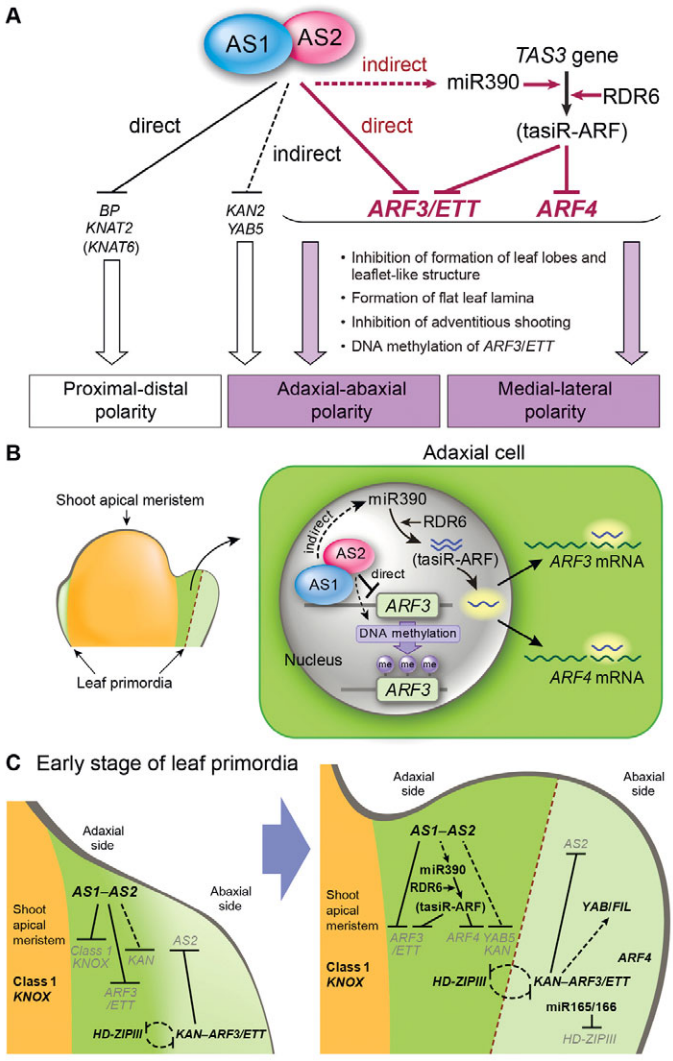


**Fig. 6. Model for regulation of *ETT* and *ARF4* by the AS1-AS2 complex in early stages of leaf primordia in *A. thaliana*.** (**A**) Summary of the roles of AS1 and AS2 in leaf development. Red lines indicate dual regulation of *ETT* by the AS1-AS2 complex, as proposed in this article. (**B**) Proposed mechanisms of AS1 and AS2 repression of *ETT* in the adaxial cells of early-stage leaf primordia. (**C**) Temporal regulation of the gene 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; Vaughn 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 *AS1* and *AS2* mediate DNA methylation of *ETT* by additional pathways parallel with regulation by *MET1*.

**Acknowledgements**

The authors thank Ms A. Takahashi, Ms K. Fukatsu, Mr K. Oga, Ms M. Takahashi and Mr Y. Yokoyama for their skilled technical assistance.

**Funding**

This work was supported by four Grants-in-Aid: Scientific Research on Priority Areas [19060003 to Y.M.]; Scientific Research (C) [21570052 and 24570061 to C.M.; 22570053 to S.K.]; and Young Scientists (B) [21710211 and 24710222 to H.T.] from the Ministry of Education, Science, Culture and Sports of Japan, and by the 'Academic Frontier' Project for Private Universities (matching fund subsidy from MEXT, 2005-2009). Deposited in PMC for immediate release.

**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.085365/-/DC1

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