Dual regulation of \textit{ETTIN (ARF3)} gene expression by AS1-AS2, which maintains the DNA methylation level, is involved in stabilization of leaf adaxial-abaxial partitioning in \textit{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 subject to the same Creative Commons License terms.

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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 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 KANAT2, 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 mediolateral 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), and mifepriste (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 designated as pETT4.9kb-GUS) (Ng et al., 2009) was digested with NolI and Ndel, 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′-CTCAATTTGCTAGCTATGATGCATACAGTGGT-3′) and AS1R1NotI (5′-GAGTCGACATGGCATCTTCTTCATGCTACATGTCATCCTTGTAGTCA-3′) and pU330-FLAG (5′-GACCCCCGGCTTGTCTATCCTGTCATGTCATCCTTGTAGTCA-3′). After digestion with BglII, the fusion construct was isolated and inserted between the BglII and NolI sites of pBSK-AS1. The resultant fusion construct was cloned into the pGreen0029 binary vector (Hellens et al., 2000) to yield pAS1:T7:AS1. The coding DNA 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′-CTAGGGACTACAAGGATCAGATGGGGATTATAGAAGACACGAGCATGATCAAGATGACATGTCATCCTTGTAGTCA-3′ and pU331-FLAGA (5′-GATCCCCGGCTTGTCTATCCTGTCATGTCATCCTTGTAGTCA-3′). Following digestion with XhoI, the fragment was inserted between the AarI and SalI sites of pAS2-TAS2, which was constructed by the insertion of the 5′ region of AS2 (nucleotides −3301 to −1), the recognition sites of AarI and SalI, and the 3′ region of AS2 (nucleotides +595 to +2868), into the Apal/EcoRV sites of pGreen0029 (Hellens et al., 2000). The resulting plasmid was designated pAS2: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 RLTKEFLQMQCGCRDRP (residues 357 to 373 of AS1) as antigen. Antibodies were affinity-purified with immobilized antigen, synthesized peptide RLTKFLEQQMGCRLDRP (residues 357 to 373 of AS1) gene by PCR with primers pU330SalI (5′-GAGTCGACATGGCATCTTCTTC-AACAAACTC-3′) and FLAGR2NotI (5′-AACTCAGGCCGGCGCTACACTTGCTCATTGTCATGTCATCCTTGTAGTCA-3′). Following digestion with XhoI, the fragment was inserted between the AarI and SalI sites of pAS2-TAS2, which was constructed by the insertion of the 5′ region of AS2 (nucleotides −3301 to −1), the recognition sites of AarI and SalI, and the 3′ region of AS2 (nucleotides +595 to +2868), into the Apal/EcoRV sites of pGreen0029 (Hellens et al., 2000). The resulting plasmid was designated pAS2: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).

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 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 Affymetrix 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 asl-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 asl-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, asl-1, as2-1 and an ectopic overexpressor of AS2 cDNA (pAS1:AS2; designated AS2-eeo) in the as2-1 mutant, we showed that 382 genes were expressed in two clusters, 3 and 6: expression was enhanced in both
arfun1 and arfun2 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 arfun1 and arfun2 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 of AS1 and/or AS2 to this region, we performed a ChIP-PCR initiation site of ET2. As shown in Fig. 1E, a region 2.8 kb upstream of the translational promoter ET2 is involved in leaf polarity regulation, we analyzed its regulation in lines with arfun1 and arfun2 (supplementary material Fig. S1B) 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 arfun1 and arfun2 plants (18-day-old T1) carrying the pETT2.3kb: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. (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), arfun1 and arfun2 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 levels of transcripts and further normalized to the respective mock-treated controls. ETT Transcript levels in 35S:AS2:GR/Col-0, 35S:AS2:GR/arfun1 and 35S:AS2:GR/arfun2-30 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 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 tasiARF pathway. We monitored the levels of miR390 and tasi-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 tasi-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.
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, KNOTTED1 and KNOTTED6 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 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 epigenonics 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 METHYLTRANSFERASE 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
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.
activities of several abnormalities of several levels: temporally, transcriptionally, post-transcriptionally. Expression, we showed here that AS1-AS2 acts to restrict leaf primordia. Although these two factors have overlapping expression, two exceptions; transcriptional regulators. All are expressed in a polar manner with establishment of adaxial-abaxial leaf polarity in Arabidopsis (Fig. 6C; Sarojam et al., 2010; Kelley et al., 2012). The mutual regulations among these genes also contribute to the establishment of adaxial-abaxial leaf polarity in Arabidopsis (Fig. 6C; Sarojam et al., 2010; Kelley et al., 2012).

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 (CWGGTD), which is a c-Myb-related sequence (CNGTDD), 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.

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