Focus Article

The complex of ASYMMETRIC LEAVES (AS) proteins plays a central role in antagonistic interactions of genes for leaf polarity specification in *Arabidopsis*

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Leaf primordia are born around meristem-containing stem cells at shoot apices, grow along three axes (proximal–distal, adaxial–abaxial, medial–lateral), and develop into flat symmetric leaves with adaxial–abaxial polarity. Axis development and polarity specification of *Arabidopsis* leaves require a network of genes for transcription factor-like proteins and small RNAs. Here, we summarize present understandings of adaxial-specific genes, *ASYMMETRIC LEAVES1* (*AS1*) and *AS2*. Their complex (AS1–AS2) functions in the regulation of the proximal–distal leaf length by directly repressing class 1 *KNOX* homeobox genes (*BP, KNAT2*) that are expressed in the meristem periphery below leaf primordia. Adaxial–abaxial polarity specification involves antagonistic interaction of adaxial and abaxial genes including *AS1* and *AS2* for the development of two respective domains. AS1–AS2 directly represses the abaxial gene *ETTIN/AUXIN RESPONSE FACTOR3* (*ETT/ARF3*) and indirectly represses *ETT/ARF3* and *ARF4* through tasiR-ARF. Modifier mutations have been identified that abolish adaxialization and enhance the defect in the proximal–distal patterning in *as1* and *as2*. AS1–AS2 and its modifiers synergistically repress both *ARFs* and class 1 *KNOXs*. Repression of *ARFs* is critical for establishing adaxial–abaxial polarity. On the other hand, abaxial factors KANADI1 (KAN1) and KAN2 directly repress *AS2* expression. These data delineate a molecular framework for antagonistic gene interactions among adaxial factors, *AS1*, *AS2*, and their modifiers, and the abaxial factors *ARFs* as key regulators in the establishment of adaxial–abaxial polarity. Possible AS1–AS2 epigenetic repression and activities downstream of *ARFs* are discussed.

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INTRODUCTION

Leaves develop as lateral organs from the peripheral zone of a shoot apical meristem (SAM) along three structural axes. A group of cells is initially patterned along the proximal–distal axis and then along the adaxial–abaxial axis. Subsequent cell proliferation along the medial–lateral axis results in flat and mediolateral symmetric leaves[1–9] (Figure 1).

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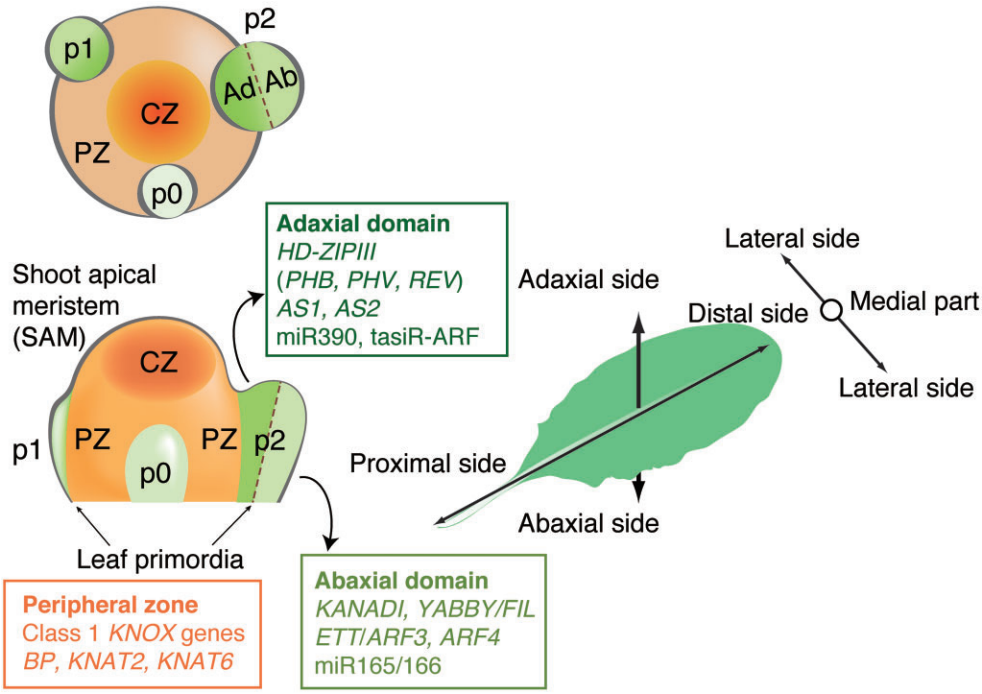

FIGURE 1 | The leaf structure develops along three axes. Developmental compartments in the shoot apex around the apical meristem and the three structural leaf axes are schematically shown on the left-hand side and in the middle, respectively (see details in text). CZ, central zone; PZ, peripheral zone; p0, primordium 0; p1, primordium 1; p2, primordium 2.

The process of leaf differentiation is a good model to study organ development from stem cells. The SAM consists of stem cells in a central zone (CZ), which divide slowly and replenish a peripheral zone (PZ) of more rapidly dividing cells, in which leaf initiation occurs[10] (Figure 1). Leaf primordia are detected as transcriptionally distinct groups of leaf founder cells before they become morphologically distinct from the SAM. This process was first clearly demonstrated as the disappearance of class-1 *KNOTTED*-like homeobox (class 1 *KNOX*) gene transcripts from the leaf primordia.[11] In dicotyledonous plants, a leaf primordium 0 (p0) is initially contained entirely within the SAM (see Figure 1), and then begins to grow outward.[12] It has been speculated that the primordium acquires adaxial–abaxial polarity in the radial dimension,[13] soon after it becomes visible, which is between the p1 and p2 stages of development. Based on the observation that if adaxial–abaxial polarity is perturbed, filamentous shaped leaves are formed, Waites and Hudson[14] proposed that cell proliferation might be induced at the boundary between the adaxial and abaxial domains, and result in the expansion of leaf lamina in the medial–lateral direction. Genetic and molecular studies of leaf development in dicotyledonous plants support their concept. The molecular mechanisms underlying the cell proliferation induced by the juxtaposition of these two domains, however, are not well understood.

As summarized in Figure 1, genes involved in the adaxial–abaxial partitioning of leaves have been isolated and characterized in *Arabidopsis thaliana*. Analyses of these genes have shown that networks of several families of transcription factor-like proteins and small RNAs must play critical roles in the specification of leaf polarity. The *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), and *REVOLUTA* (*REV*) genes, which encode class III homeodomain-leucine zipper (HD-ZIPIII) proteins, determine adaxial cell fate,[15] The accumulation of their transcripts in the adaxial domain is a consequence of their degradation in the abaxial domain by microRNA165 (miR165) and miR166 (miR165/166).[16] The *ASYMMETRIC LEAVES1* (*AS1*) and *AS2* genes, which encode nuclear proteins with the MYB (SANT) domain[17] and the plant-specific AS2/LOB domain (https://www.arabidopsis.org/browse/genefamily/AS2.jsp), respectively[18–20] (Figure 2(a)) were initially identified as factors involved in the formation of symmetric flat lamina of leaves.[21] It has recently been shown, however, that they are related to the formation of proper morphology along three leaf axes including the adaxial–abaxial axis, which will be mainly discussed in this article.

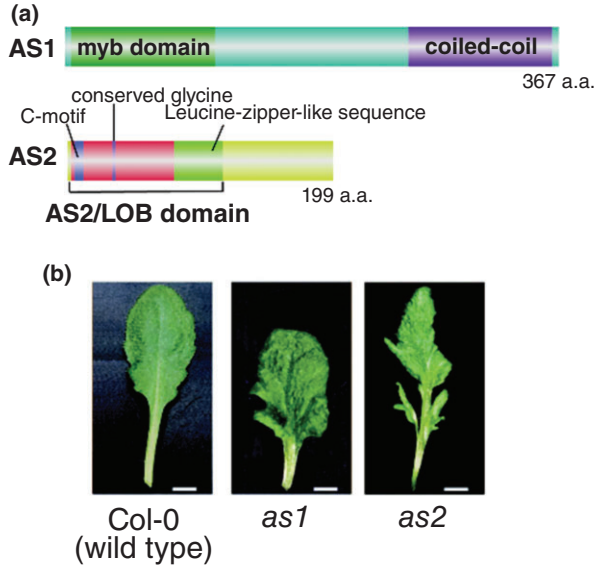

FIGURE 2 | (a) Domain organization of AS1 and AS2 proteins. Both are nuclear proteins. AS2 belongs to the AS2/LOB plant-specific protein family (42 members are designated as AS2 and ASL1–ASL41).[18,20] (b) Phenotypes of *as1* and *as2* leaves. Both *as1* and *as2* show pleiotropic phenotypes including asymmetrically curled leaf blades, asymmetric lobes, asymmetric secondary vein patterns, less prominent midveins, plump and swelled leaf laminas, and shorter petioles than seen in wild type leaves. Asymmetric leaflet-like structures are formed in *as2*. Photograph (*as2*) is reproduced from Ref 5 (*Development* 2001, 128:1771–1783).

Members of the *KANADI* (*KAN*) gene family, which encode proteins with the GARP domain, and members of the *YABBY* (*YAB*)/*FIL* family (https://www.arabidopsis.org/browse/genefamily/C2C2YABBY.jsp), which encode proteins with a zinc finger and a HMG box-like domain are involved in the specification of abaxial cell fate in the leaf lamina.[6–9] In addition to the abaxial development, it is also suggested that *YAB* genes are involved in developing the expanding flat leaves with diverse systems characterized by more lamina-specific genetic programs that are related to marginal auxin flow and activation of a maturation schedule directing determinate growth.[22] *ETTIN/AUXIN RESPONSE FACTOR3* (*ETT/ARF3*) and *ARF4* (https://www.arabidopsis.org/browse/genefamily/ARF.jsp) also specify both abaxial cell fate and lateral growth of leaf lamina.[23] This result suggests that the lateral growth of the lamina could be related to the determination of adaxial–abaxial identity as proposed previously.[14] Transcripts of both *ETT/ARF3* and *ARF4* are specifically degraded by the small RNA tasiR-ARF in the presumptive adaxial domain, contributing to the determination of the adaxial cell fate.[24,25]

The idea that leaf polarity is specified by antagonistic interactions between adaxial and abaxial genes was proposed on the basis of genetic and expression analyses of these genes.[26] Such expression patterns change during leaf development. During this process, both adaxial and abaxial promoting genes are initially expressed throughout the primordium (see p0–p1 in Figure 1), and subsequently their expression patterns are restricted to their respective complementary domains (p2). The patterning of expression of the polarity genes is generated by the mutually exclusive actions of their protein products. For example, expression of the abaxial gene *FIL/YAB* is abolished by the ectopic expression of *PHB* (*HD-ZIPIII* adaxial gene).[27] Although leaf regions expressing *PHB* and *KAN* messenger RNAs (mRNAs) are mutually exclusive,[15,28] and these gene families act genetically in an antagonistic manner during embryo patterning,[29] it has not been clearly demonstrated that *KAN* regulates *HD-ZIPIII* expression directly.

Recently, Nakata et al.[30] showed that *PRESSED FLOWER/WUSCHEL-RELATED HOMEOBOX3* (*WOX3*) and *WOX1* (https://www.arabidopsis.org/browse/genefamily/wox.jsp), which are expressed in the middle domain between the adaxial and abaxial domains, function redundantly in lateral-specific lamina outgrowth and leaf margin-specific cell fate and, furthermore, that expression patterns of the two *WOX* genes are negatively and positively regulated by the *KAN* and *YAB* genes, respectively. They also propose a three-domain model, in which these *WOX* genes would coordinate adaxial/abaxial patterning in cooperation with adaxial- and abaxial-specific regulators, including the *ASYMMETRIC LEAVES2* (*AS2*) and *YAB3*. *YUCCA* genes, responsible for auxin biosynthesis, are expressed in response to the juxtaposition of adaxial and abaxial domains, which is responsible at least in part for leaf margin expansion.[31]

AS1 and *AS2*, both of which are referred as to as adaxial genes and exhibit similar laminar abnormalities, repress the expression of abaxial genes, such as *KAN2*, *YAB5*, *ETT/ARF3*, and *ARF4*, but do not affect the *HD-ZIPIII* family genes,[32] suggesting that they are involved in the antagonistic interactions between genes that specify adaxial–abaxial polarity. The direct repression of *AS2* by KAN was first reported by Wu et al.[33] Our group has recently reported the direct repression of *ETT/ARF3* by transcriptional gene silencing (TGS) through AS1–AS2 and indirect repression of both *ARF3* and *ARF4*, a redundant member of the *ARF* gene family, by post TGS (PTGS) through AS1–AS2 functions.[34] These results provide a molecular framework for the antagonistic interaction of genes involved in adaxial–abaxial specification. In this article, we will

overview the recent results on molecular mechanisms for the opposing interplay of polarity-related genes by AS1 and AS2 and discuss prospects for a novel epigenetic system of gene repression to guarantee the polarity specification necessary for leaf development.

CHARACTERIZATION OF ADAXIAL-SPECIFIC *AS1* AND *AS2* GENES

The *PHANTASTICA* (*PHAN*) gene of *Antirrhinum majus* is involved in the growth and adaxial–abaxial determination of lateral organs and its expression is required early in the establishment of the proximal–distal axis.[2,14] Because plants with a mutation in *PHAN* are known to generate abaxialized filamentous leaves only when grown at 15–17 °C[2,14] or in the *handlebars* (*hb*) mutation background, it is proposed that a cold-sensitive pathway and some other gene might be redundantly involved in the adaxial–abaxial determination of leaves together with *PHAN* (see the later section of 'Modifier mutations' of *Arabidopsis*).[35] In the *as1* mutant in *A. thaliana*, the *PHAN MYB* ortholog is disrupted.[17]

Both *as1* and *as2* mutants exhibit pleiotropic phenotypes[5,36,37] (Figure 2(b)). These mutants produce petioles and leaf blades that are much shorter than those of the wild type, in addition to asymmetrically lobed and downwardly curled leaf blades, which are bilaterally asymmetric. Furthermore, *as1* and *as2* also often generate leaflet-like structures from petioles in asymmetric positions and fail to produce a thick and distinct midvein; and *as2* often generates leaflet-like structures from petioles in asymmetric positions. Higher-ordered veins are asymmetric and simplified. The observation that the leaf laminas of *as1* and *as2* are often plump and swelled at their base implies that adaxial development in the leaves of these mutants is slightly diminished.[5] Thus, the *AS1* and *AS2* genes are involved not only in the symmetric development along the mediolateral axis, but also in development along the adaxial–abaxial axis and the proximal–distal axis. In addition, expression levels of many genes, including both class-1 *KNOX* genes (*BP*, *KNAT2*, *KNAT6*) and abaxial-determinant genes, such as *ETT/ARF3*, *KAN2* and *YAB5*, are elevated.[5,18,38]

Both *AS1* and *AS2* transcripts accumulate in the early stage of above-ground organ primordia, and *AS1* and *AS2* expression sites become restricted to middle/inner regions and the adaxial epidermis of cotyledonary and leaf primordia. AS2 transcripts are also accumulated in the columella root cap.[32] After leaf maturation, the expression levels of these genes are reduced.[32] AS2-fused YFP (AS2-YFP) proteins are localized to subnuclear bodies[39–41] adjacent to the nucleoli in leaf cells, called AS2 bodies, and some are also dispersed in the nucleoplasm.[39,41] GFP-fused AS1 proteins are located as speckles in the nucleoplasm[39–41] and are also concentrated in the AS2 bodies by an AS2-dependent process.[39,41] AS1 and AS2 form the AS1–AS2 complex,[42,43] which represses the expression of two class 1 *KNOX* genes, *BP* and *KNAT2*, by binding to their respective promoter regions, showing that these *KNOX* genes are direct targets of AS1–AS2.[42] In addition, AS1–AS2 directly represses *ETT/ARF3* by binding to its promoter region.[34]

Although genes that are predicted to encode AS1 orthologs and members of the AS2/LOB protein family are detected in genome databases of many plant species, genes that might encode amino acid sequences entirely homologous to the AS2 sequence are not detected in rice genome databases, and even complementary DNA (cDNAs) encoding AS2 orthologs have yet to be reported from monocotyledonous plants. Although AS2 homologues have been predicted to be present in various dicotyledonous plants, their roles in plants other than *Arabidopsis* are not yet intensively studied.

PROXIMAL–DISTAL POLARITY DEVELOPMENT OF LEAVES BY AS1–AS2

Genes involved in the formation of proximal–distal polarity were first identified in maize. While leaves of dicotyledonous plants are composed of stipule, petiole, and leaf blade along with proximal–distal axis, monocotyledonous plants such as maize and rice develop other distinct leaf features: the sheath in the proximal region of the leaf and the blade in its distal region. The sheath and blade are separated at their boundary by the auricle and ligule, which are not present in leaves of dicotyledonous plants (http://www.fsl.orst.edu/forages/projects/regrowth/printsection.cfm?title=Grass Structures). Recessive mutants of the *rough sheath2* (*rs2*) gene of maize, an ortholog of *PHAN* and *AS1*,[44,45] exhibit a disruption of the blade-sheath boundary owing to disorganized cell growth and acropetal ligule displacement, and the semi-bladeless phenotype of leaves.[46] In *rs2* mutants, class 1 *KNOX* genes are ectopically expressed, a condition that is also observed in some dominant mutants exhibiting phenotypes similar to those of *rs2*. Thus, *rs2* is involved in the proximal–distal patterning of maize leaves through repression of class 1 *KNOX* genes.[47]

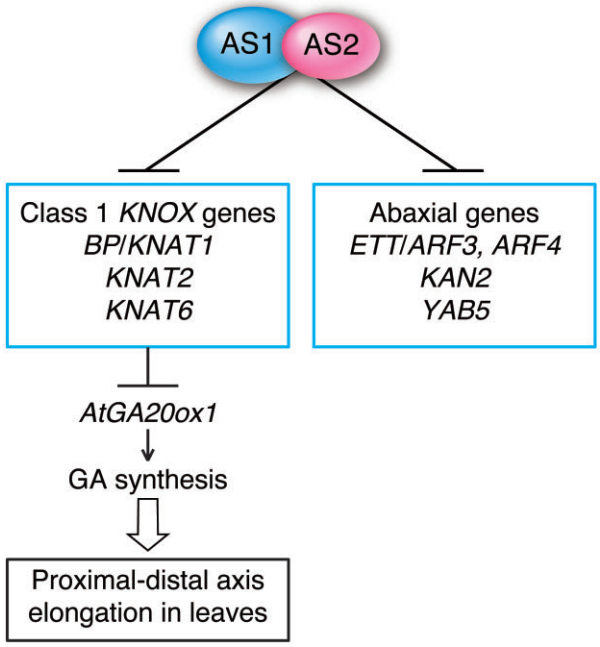

FIGURE 3 | Roles of the AS1–AS2 complex in the regulation of class 1 *KNOX*, *ETT/ARF3* and *ARF4* genes in early stages of leaf primordia in *Arabidopsis thaliana*. The introduction of *bp knat2 knat6* triple mutations into *as1-1 or as2-1* efficiently suppressed the phenotypes of short petiole and leaf blade seen in Figure 2(b).[48]

AS1 and *AS2* of *A. thaliana* are also involved in regulation of the proximal–distal development of leaves through repression of class 1 *KNOX* genes (Figure 3). Although petiole and leaf lengths are markedly reduced both in *as1* and *as2*, the extent of the reduction is more severe in *as1*.

To investigate effects of the elevated expression of class 1 *KNOX* genes on the phenotypes of *as1* and *as2* mutants, a number of studies have been performed using over- and ectopic-expression systems of class 1 *KNOXs* under the control of the 35S promoter of Cauliflower mosaic virus. Overexpression of *KNOX* genes in tobacco and *A. thaliana* plants repressed transcription of the gibberellin-synthetic (GA-synthetic) genes that encode GA-20 oxidase,[49,50] and application of GA partially suppressed the abnormal phenotypic features of *PHANTASTICA*-antisense transgenic tobacco plants.[51] Analyses of multiple loss-of-function mutants of *KNOX* genes (*bp knat2 knat6*) in *as1* and *as2* backgrounds show that the formation of shorter petioles and leaf blades is due to repression of GA-synthetic genes by the upregulation of *BP/KNAT1*, *KNAT2*, and *KNAT6*[48] (Figure 3). Thus, elevated expression of *KNOXs* is responsible for limited numbers of *as1* and *as2* phenotypes including petiole and lamina sizes, the less prominent midvein, and the lower potential of root regeneration from leaf sections in *in vitro* culture. The formation of asymmetric leaf lobes, leaf curling, leaflet-like structures from petioles, and the increased potential of shoot regeneration are, however, not due to upregulation of class 1 *KNOXs*.

PHAN in *Antirrhinum* is also involved in elaboration of the proximal–distal axis as well as the adaxial–abaxial polarity in leaves.[2] *NSPHAN* of *Nicotiana sylvestris* is also proposed to be involved in proximal–distal development.[47] Taken together, the *KNOX*-repressive systems mediated by *AS1* orthologs (*PHAN* and *RS2*), which appear to be involved in the proximal–distal polarity patterning, might be conserved at least in the plants mentioned in this section. Nevertheless, roles of AS2 orthologs in such patterning have not been determined in these plants other than *Arabidopsis*.

ADAXIAL–ABAXIAL POLARITY SPECIFICATION OF LEAVES BY AS1–AS2

Molecular Roles of AS1–AS2: Repression of Abaxial Genes

Gene expression analyses of *as1* and *as2* show that transcript levels of several abaxial side-specific genes (*ETT/ARF3*, *KAN2*, *YAB5*) are significantly increased, whereas those of *HD-ZIPIII* do not change.[32] These results suggest that AS1 and AS2 directly or indirectly repress expression of the abaxial-specific genes (Figure 3). In addition, systematic molecular and genetic analyses have identified a target gene, *ETT/ARF3*, which encodes an abaxial factor acting downstream of the AS1–AS2 complex.[34] As schematically summarized in Figure 4, the AS1–AS2 complex represses *ETT/ARF3* by the direct binding of AS1 to the *ETT/ARF3* promoter and also indirectly induces accumulation of miR390 and tasiR-ARF, which negatively regulate the expression of both *ETT/ARF3* and *ARF4*. Thus, the complex dually represses the expression of *ETT/ARF3*. Several abnormalities of *as2* plants are slightly suppressed by the introduction of an *ett* or *arf4* single mutation into *as2* plants. The introduction of *ett arf4* double mutations into *as2* efficiently suppresses these asymmetric leaf phenotypes[34] (Figure 5(a)). These results are consistent with the observation that overexpression of a tasiR-ARF-insensitive *ETT/ARF3* cDNA yields *as2*-like phenotypes.[52] Similarly, some lamina phenotypes of *as1* were also rescued by the introduction of *ett arf4* (Figure 5(a)). The phenotype of wrinkled lamina with patches of abaxialized cells on the adaxial surface, which indicates a slight

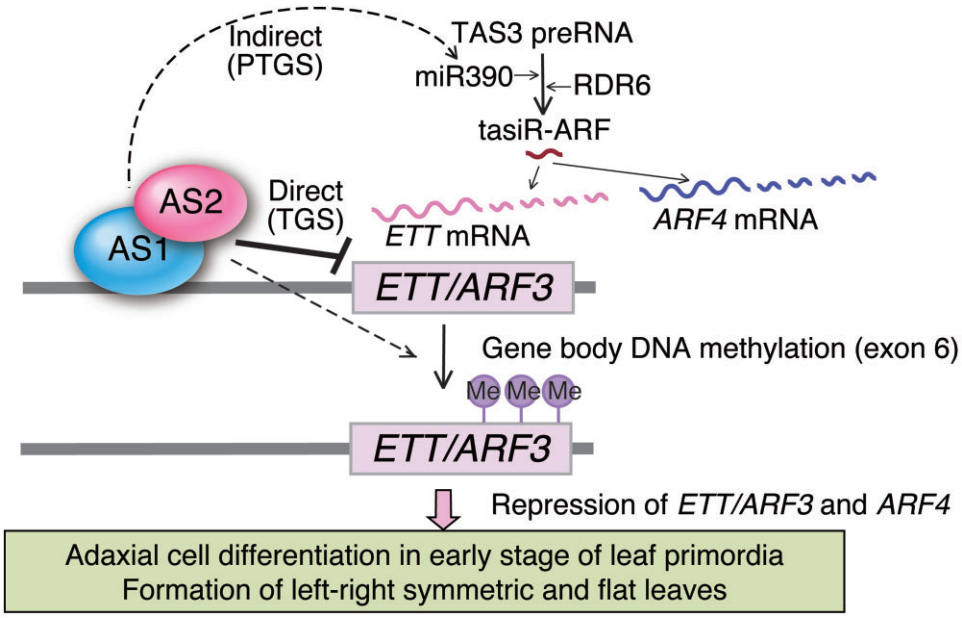

FIGURE 4 | Dual regulation of *ETT*/*ARF3* gene expression, including by the possibly epigenetic system of AS1–AS2. The AS1–AS2 complex represses *ETT*/*ARF3* directly, and *ETT*/*ARF3* and *ARF4* indirectly, via stimulating the miR390 and tasiR-ARF pathway. In addition, *AS1* and *AS2* maintain gene-body DNA methylation of the *ETT*/*ARF3* gene. Solid lines indicate direct regulation and dashed black lines indicate indirect regulation.

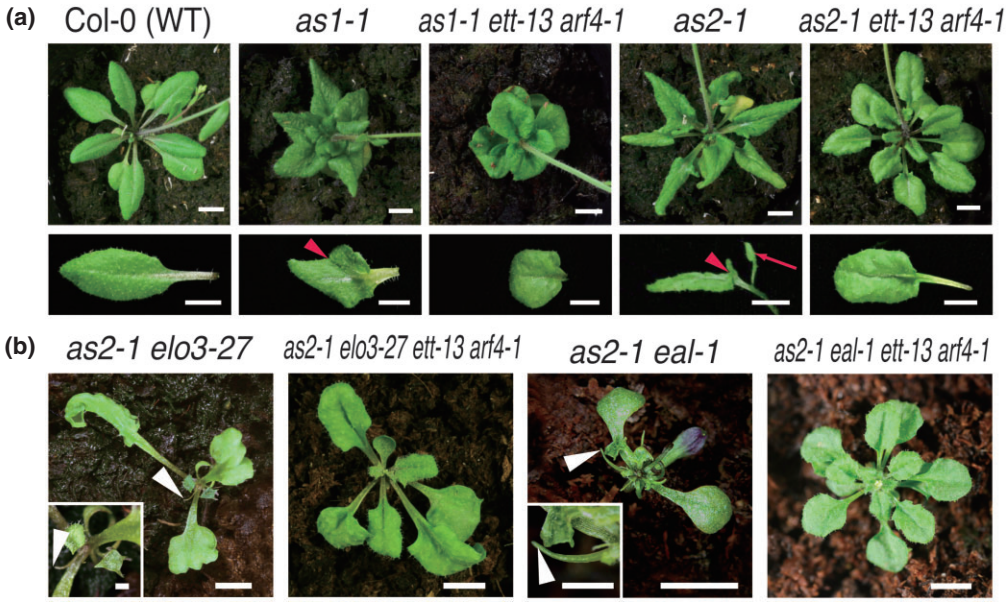

FIGURE 5 | (a) The *ett* and *arf4* mutations suppressed major leaf phenotypes of *as1-1* and *as2-1*. Representative gross morphology of 40-day plants and magnified views of their leaves. Gross morphology of Col-0 (wild type), *as1-1*, *as1-1 ett-13 arf4-1*, *as2-1*, and *as2-1 ett-13 arf4-1* plants is shown. The genotype of each plant is indicated. Red arrowheads indicate leaf lobes and the arrow indicates a leaflet-like structure. The introduction of *ett arf4 double* mutations into *as1-1 or as2-1* efficiently suppressed the phenotypes of asymmetrically curled leaf blades, asymmetric lobes, and plump and swelled leaf laminas in both mutants[34] in Figure 2(b). Scale bars: 5 mm (upper) and 2 mm (lower). (b) Gross morphology of typical double mutants (*as2-1 elo3-27* and *as2-1 eal-1*/*bob1*). Introduction of *ett* and *arf4* mutations into the double mutants efficiently suppressed the abaxialized leaf phenotypes to form flat symmetric leaves. See details of modifier mutations in Table 1. Scale bars: 5 mm. Plants were photographed at 28 days after sowing. White arrowheads indicate filamentous leaves. Scale bars: 5 mm. Higher magnification views of filamentous leaves are shown. Scale bars: 1 mm in higher magnification views. Photographs (a) and (b) are reproduced and modified from Ref 34 (*Development* 2013, 140:1958–1969) and Ref 69 (*Plant Cell Physiol* 2013, 54:418–431), respectively.

increase in abaxialization,[14,53] was also rescued in both *as1* and *as2* by the introduction of *ett arf4*.[34] These results suggest that several leaf abnormalities, including those related to adaxial–abaxial polarity defects in *as1* and *as2* plants, result from elevated expression of the abaxial genes *ETT* and *ARF4* (Figure 4). Analyses of modifier mutations of *as1* and *as2* have further confirmed that repression of these *ARFs* by AS1–AS2 is important for the adaxialization of leaves. Although expression levels of *KAN2* and *YAB5* are increased in *as1* and *as2*, they are indirectly regulated by AS1–AS2.[34]

Although the systems whereby tasiR-ARFs regulate *ARF3* expression are conserved in both *Arabidopsis* and maize plants, the contribution of tasiR-ARFs to adaxial–abaxial patterning in *Arabidopsis* seems to be different from that in maize; the extents of adaxial defects in mutations in tasiR-ARF biogenesis components of *Arabidopsis* are subtle as compared with those of maize.[13] This might be due to difference in the involvement of *AS1* in repressing *ARF3* expression in *Arabidopsis* from that of *RS2* in repressing *ARF3* in maize. Recently, loss-of-function mutants of small RNA biogenesis components (*RDR6*, *SGS3*, *AGO7*, and *DCL4*) in tomato (*Solanum lycopersicum*) have been isolated. In severe cases, they generate shoestring leaves that lack leaf blade expansion (*wiry* leaves).[54] In the tomato mutants, levels of tasiR-ARFs are reduced and *ARF3* and *ARF4* are upregulated, suggesting that the repressive system of *ARF3* and *ARF4* regulation by tasiR-ARFs is also conserved in the pathway for adaxial–abaxial specification in leaves of tomato; increased activity of either of *ARFs* phenocopies results in *wiry* leaves. Interestingly, overexpression of these *ARFs* in *Arabidopsis*, tobacco (*Nicotiana tabacum*), and potato (*Solanum tuberosum*), however, fails to produce *wiry* leaves, suggesting that such a response in tomato is distinct from those in other dicotyledonous plants. The tomato system of adaxial–abaxial specification by tasiR-ARFs appears to be somewhat similar to the developmental control of adaxial–abaxial patterning by the tasiR-ARFs in maize.

Modifier Mutations Enhancing Leaf Polarity Defects of *as* Mutants

Many mutations that enhance leaf polarity defects of *as1* and *as2* have been isolated and characterized, which is reminiscent of the presence of the cold-sensitive pathway in the original *phan* mutant of *Antirrhinum* and *handlebars* as the enhancer mutation of *phan*.[2,35] The causative genes are designated as modifiers of *AS1* and *AS2*[34,69] and, as listed in Table 1,[24,30,34,39,58,59,62–64,69–72,74–79,81,82,84,88–90,93,95] they include those for biogenesis of tasiR-ARF, biogenesis of ribosomes, chromatin modification and nucleosome assembly proteasome-mediated protein degradation, genomic stability, and cell proliferation. Prominent phenotypes in almost all double mutants with *as2* and a modifier mutation involve the generation of filamentous leaf-like organs (Figure 5(b)), which are surrounded by an abaxialized epidermis and possess no or markedly premature vascular tissues. Double mutations in *PRESSED FLOWER/WUSCHEL-RELATED HOMEOBOX3* (*WOX3*) and *WOX1* also cause the formation of severely abaxialized filamentous leaves in the *as2* background.[30,89]

In the double mutants that have been examined, transcript levels of several abaxial-specific genes (*KAN2*, *YAB5*, *ETT/ARF3*, *ARF4*) as well as class 1 *KNOX* genes are markedly increased; these genes are upregulated in the *as2* single mutant and slightly upregulated in some of the modifier single mutants. When the double mutations of *ETT/ARF3* and *ARF4* (see Figure 4), are introduced into double mutants with *as2* and one of the modifier mutations, such as *elo3* and *bob1/eal-1*, the phenotype of abaxialized filamentous leaves is restored to flat and expanded shapes[34,69] (Figure 5(b)). These results show that the upregulation of these *ARF* genes in the double mutants is responsible for the disappearance of adaxial specification of the mutants, which suggests that repression of these *ARF* genes by the synergistic action of AS1–AS2 and modifier proteins is critical for proper development of the adaxial domain.

How can the modifiers and AS1–AS2 synergistically repress expression of *ARFs* in a wild type plant? As mentioned in the previous section, *ETT/ARF3* expression is regulated dually by AS1–AS2-dependent TGS and tasiR-ARF-mediated PTGS through AS1–AS2, and *ARF4* is regulated by the PTGS. Therefore, the synergistic repression of these *ARFs* is achieved by the two independent pathways, AS1–AS2 and factors involved in small RNA biogenesis such as *RDR6*, *AGO7*, and *DCL4*, as illustrated in Figure 4. Molecular mechanisms for the prominent repression by other modifiers and AS1–AS2 have yet to be elucidated, but they might be involved in such repression through independent pathways[69,70,72,90] (Figure 6). It might be worthwhile, however, to stress that modifier mutations so far identified are weak alleles of genes that are essential for cell viability or, conversely, strong alleles of one of the functionally redundant members of such essential gene families. In addition, it is also interesting to note that most of the proteins encoded by such modifier genes are localized in the nucleus or nucleus-related structures or compartments, such

TABLE 1 | *Arabidopsis* Gene Mutations, Which Act as Modifiers Enhancing Leaf Adaxial–Abaxial Abnormalities in *as1* and *as2*

1. Gene (Mutation)	2. AGI Code	3. Protein	4. Cellular Process and Status	5. Subcellular Localization	6. References
I. **Biogenesis of small RNA**					
RNA-DEPENDENT RNA POLYMERASE6 (*rdr6*/*sde1*/*sgs2*)	AT3G49500	RNA-dependent RNA polymerase	Duplication of TAS3 mRNAs; biogenesis of ta-siRNA	Cytoplasm,[55,56] nucleus[57]	24, 58
ARGONAUTE7 (*ago7*/*zip*)	AT1G69440	ARGONAUTE family protein: RNA slicer	Biogenesis of miR390 for ta-siRNA production	Cytoplasm[55]	24, 59
SUPPRESSOR OF GENE SILENCING3 (*sgs3*)	AT5G23570	Unknown	Biogenesis of siRNA, stabilization of ta-siRNA	Cytoplasm[55,56]	24, 59
DICER-LIKE4 (*dcl4*)	AT5G20320	DICER-LIKE protein: RNase III-like enzyme	Processing of ta-siRNA intermediates	Nucleus[60,57]	24, 59
ARGONAUTE1 (*ago1*)	AT1G48410	ARGONAUTE family protein: RNA slicer	Recruit of miRNA and siRNA to mRNAs to be degraded	Nucleus (D-body) and cytoplasm[61]	59, 62, 63
II. **Chromatin modification and remodeling**					
PICKLE (*pkl*/*gymnos*)	AT2G25170	Chromodomain helicase DNA-binding (CHD3) family protein	Component of chromatin remodeling complex SWI/SNF		64
SERRATE (*se*)	AT2G27100	C2-H2-type zinc finger protein	miRNA-mediated gene expression	Nucleus[65]	64
HDT1 (*hdt1*/*hd2a*/*hda3*)	AT3G44750	Histone deacetylase (plant-specific class)	Deacetylation of nucleosomal histone H3, transcription of rDNAs	Nucleolus[66,39,67]	39
HDT2 (*hdt2*/*hd2b*)	AT5G22650	Histone deacetylase (plant-specific class)	Deacetylation of nucleosomal histone H3, transcription of rDNAs	Nucleolus[66,39,67]	39
ELONGATA3 (*elo3*/*east1*); *ELO2* (*elo2*/*elp1*/*abo1*); *ELONGATOR PROTEIN2* (*elp2*)	AT5G50320; AT5G13680; AT1G49540	Histone acetyltransferase; scaffold proteins	Core subcomplex of holo-elongator; stimulation of transcriptional elongation; DNA replication	Nucleus (predominant) and cytosol (lesser extent)[68]	69–71
ELO1 (*elo1*/*elp4*); *ELP5* (*elp5*); *ELP6* (*elp6*)	AT3G11220; AT2G18410; AT4G10090		Accessory subcomplex of holo-elongator; stimulation of transcriptional elongation; DNA replication		70
ELONGATA4/*DRL1* (*elo4*/*drl1*)	AT1G13870	Associated protein of elongator complex	Stimulation of transcriptional elongation; DNA replication		70, 71
FASCIATA1 (*fas1*); *FAS2* (*fas2*)	AT1G65470; AT5G64630	H3 and H4 histone chaperone	p150 subunit of chromatin assembly factor-1 (CAF-1); p60 subunit of CAF1; chromatin replication		71, 72

TABLE 1 | Continued

1. Gene (Mutation)	2. AGI Code	3. Protein	4. Cellular Process and Status	5. Subcellular Localization	6. References
III. **Ribosomal protein (or biogenesis of ribosomes)**					
RPL10a (*rpl10a*/*pgy1*); *RPL9c* (*rpl9c*/*pgy2*); *RPL5a* (*pgy3*/*ae6*/*oli5*); *RPL28a* (*ae5*); *RPL24b* (*rpl24b*/*stv1*); *RPL5b* (*rpl5b*/*oli7*)	AT2G27530; AT1G33140; AT3G25520; AT2G19730; AT3G53020; AT5G39740	L10a; L9; L5; L28e; L24b; L5b	Subunits of ribosome; components of pre-rRNA-protein complex	Cytoplasm, nucleus, and nucelolus[73]	74–77
RPL4d (*rpl4d*); *RPL7b* (*rpl7b*); *RPL18c* (*rpl18c*); *RPL38b* (*rpl38b*); *RPL39c* (*rpl39c*); *RPS6a* (*rps6a*); *RPS21b* (*rps21b*); *RPS24b* (*rps24b*); *RPS28b* (*rps28b*); *RPS15ab* (*rps15ab*); *RPL27ac* (*pgy6*/*rpl27ac*); *APICULATA2*/*RPL36AB* (*api2*); *RPL36aA* (*rpl36aa*)	AT5G02870; AT2G01250; AT5G27850; AT3G59540; AT4G31985; AT4G31700; AT3G53890; AT5G28060; AT5G03850; AT2G19720; AT1G70600; AT4G14320; AT3G23390	L4d (L1) family; L30/L7 family (translational regulation); L18e (L15) superfamily; L38e family; L39 family; S6; S21e; S24e; S28; S15AB; L18e/L15 superfamily; L44e	Subunits of ribosome; components of pre-rRNA-protein complex	Cytoplasm, nucleus, and nucelolus[73]	76–79
APUM23 (*apum23*)	AT1G72320	Pumillio protein containing PUF domain	Pre-rRNA processing and rRNA maturation	Nucleolus[80]	81
IV. **DNA replication and repair**					
TEBICHI (*tebichi*)	AT4G32700	A homologue of Drosophila MUS308 and mammalian DNA polymerase	Repair at damaged DNA		82
ABA OVERLY SENSITIVE4 (*abo4*)	AT1G08260	POL2A, DNA polymerase epsilon catalytic subunit	Interaction with PCNA; DNA-directed DNA polymerase		71
ASYMMETRIC LEAVES1/2 ENHANCER7 (*ae7*/*duf59*)	AT1G68310			Nucleus and cytoplasm[83]	84
V. **Proteasome**					
RPN8a (*asymmetric leaves enhancer3*/*rpn8a*); *RPT2a* (*hir-2*/*rpt2a*); *PBE1* (*pbe1*); *RPT5a* (*rpt5a*); *RPT1a* (*rpt1a*); *RPN9a* (*rpn9a*); *RPT4a* (*rpt4a*); *PAD1* (*pad1*)	AT5G05780; AT4G29040; AT1G13060; AT3G05530; AT1G53750; AT5G45620; AT5G43010; AT3G51260	26S proteasome subunit; 20S β subunit; one of the six AAA-ATPases of the proteasome; proteasome component domain; 20S proteasome α subunit	Component of 26S or 20S proteasome	Endoplasmic reticulum and golgi (RPT2a),[85] cytoplasm and nucleus[86,87]	88

TABLE 1 | Continued

1. Gene (Mutation)	2. AGI Code	3. Protein	4. Cellular Process and Status	5. Subcellular Localization	6. References
VI. **Transcription factors**					
PRESSED FLOWER (*prs/wox3*); *WOX1*(*wox1*)	AT2G28610; AT3G18010	WUSCHEL-related homeobox proteins	Transcription	Nucleus	30, 89
VII. **Others**					
BOBBER1 (*bobber1/enhancer of asymmetric leaves1*)	AT5G53400	A noncanonical small heat shock protein (HSP20-like chaperone); NudC domain protein	Protein folding	Cytoplasm[90,91]	69, 90
ANGUSTIFOLIA3 (*an3*)	AT5G28640	Growth regulating factor1 (GRF1) INTERACTING FACTOR	Transcriptional coactivator	Nucleus[92]	93
VIII. **Plastid genes**					
EMBRYO DEFECTIVE DEVELOPMENT1 (*edd1/ddm1*)	AT3G48110	Glycine-tRNA ligase	Glycil tRNA aminoacylation in mitochondria and chloroplasts	Chloroplast and mitochondrion[94]	95
SCABRA3 (*sca3*)	AT2G24120	DNA-directed RNA polymerase	Transcription in plastids and mitochondria	Chloroplast and mitochondrion[96]	95

FIGURE 6 | Model for repression of *ETT/ARF3* and *ARF4* by the AS1–AS2 complex and modifiers in the early stage of leaf primordia in *Arabidopsis thaliana*. Such repression events are crucial for the establishment of adaxial–abaxial polarity and then cell division and growth along the medial–lateral axis. Class 1 *KNOX* genes are similarly repressed by AS1–AS2 together with modifier genes, although that is not depicted in this figure.

as nucleoli and spindles for chromosome separation (Table 1). Only two genes, *WOX1* and *WOX3*, for conventional transcription factor-like proteins are identified as modifiers.

It should be interesting to solve the question of how AS1–AS2, which is localized to nuclear bodies adjacent to the nucleolus, might repress coordinately *ETT/ARF3*, *ARF4*, and class 1 *KNOX* genes with modifier proteins, after they might complete roles in polarity development of leaves. As many modifiers are localized to the nucleus or nuclear compartments, they function in certain nuclear processes to repress directly or indirectly the expression of *KNOXs* and *ARFs*. In cases where modifiers might be involved in unidentified nuclear processes, including such known processes as chromatin assembly and ribosome biogenesis mediated by some modifiers, any gene-repressive functions of AS1–AS2 must be associated with such unidentified processes.

Genes Downstream of the AS-Abaxial Factor Pathway

Transcript levels of *Kip-related protein2* (*KRP2*), *KRP5*, and *Isopentenyltransferase3* (*IPT3*) increase on the *as2* and modifier (*eal* and *elo3*) backgrounds, and such upregulation events are canceled by the introduction of an *ett arf4* double mutation into *as2*.[69] These results suggest that expression of *KRP2*, *KRP5*, and *IPT3* genes was negatively controlled by AS1–AS2

through repression of *ARF3/ETT* and *ARF4* functions in the wild type plant. *KRP2* and *KRP5* of *A. thaliana* encode cyclin-dependent kinase inhibitors (CKIs),[69,97] which interact with CDKs to inhibit their kinase activities and act as key regulators of cell cycle progression.[98] It is possible that cell proliferation required for leaf formation might be achieved by the proper repressive control of *KRP2* and *KRP5* expression by AS1–AS2.

The *IPT* genes encode adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in *A. thaliana*.[99–101] Among members of the *IPT* family, *IPT3* is expressed throughout a plant including the shoot apex and leaves.[102] These data predict that the endogenous level of cytokinin might increase around the SAM in *as1* and *as2* mutants, which might affect developmental states of cells in the leaf primordia of these mutants.

These results suggest that the AS1-AS2-ETT pathway plays a critical role in controlling the cell cycle progression and the cytokinin level at the shoot apex for leaf growth and development. The relationship between the control of these downstream genes and adaxial development of leaves by AS1–AS2, however, has been unknown.

POTENTIAL EPIGENETIC REGULATION BY AS1–AS2

The AS1–AS2 complex targets the *cis* elements in the promoters of *BP* and *KNAT2*.[42] AS1 and maize RS2, an AS1 ortholog, interact with the plant HIRA proteins which is predicted to be a histone chaperone that maintains *KNOX* gene silencing.[103] Polycomb-repressive complexes (PRCs) ensure the correct spatiotemporal expression of numerous key developmental regulators. Recently, it was shown that the AS1–AS2 complex physically interacts with PRC2 and recruits this complex to the homeobox genes *BP* and *KNAT2* to stably silence in differentiating leaf cells.[104] This recruitment mechanism resembles the Polycomb response element-based recruitment of PRC2 originally defined in flies and provides the first such example in plants. These findings reveal a conserved paradigm to epigenetically regulate homeobox gene expression during development.

It has also been shown that levels of DNA methylation in exon 6 of *ETT* were depressed in both *as1* and *as2* mutants.[34] It was reported that over one third of expressed genes in *A. thaliana* contain DNA methylation within their transcribed regions,[105] and loss of such methylation results in enhanced levels of transcription.[106] Recently, it has been verified that DNA demethylation increases *ETT* expression in a mutant for *MET1*.[107] Levels of *ETT* transcripts increase in shoot apices of *met1*.[34] There are clear parallel relationships between levels of the *ETT* gene-body methylation and its transcript levels in *as1* and *as2*,[34] implying the involvement of AS1–AS2 in epigenetic control through DNA methylation.

CONCLUSION

In *Arabidopsis* and some other dicotyledonous plants, development of the adaxial domain requires two types of genes: genes for the HD-ZIPIII protein family and those for the AS1–AS2 complex. In the present review, we have summarized two characteristic features of the AS1–AS2-mediated adaxialization (Figures 6 and 7): (1) This complex dually represses expression of

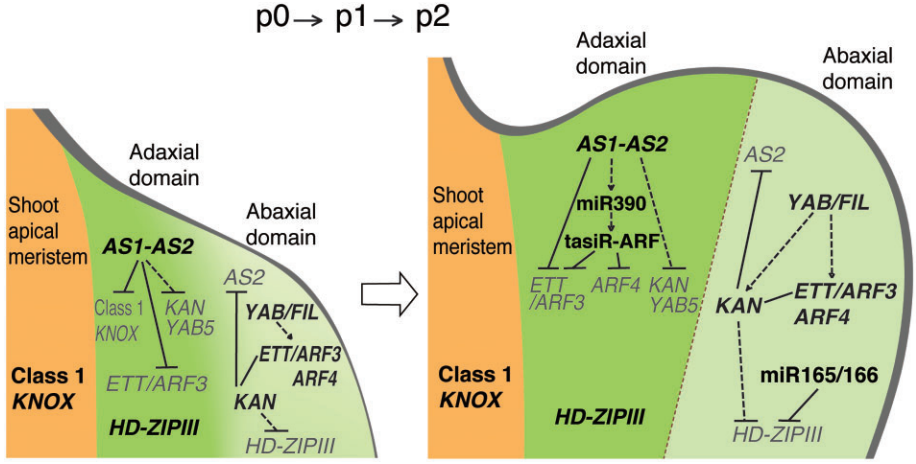

FIGURE 7 | AS1–AS2 plays a central role in the antagonistic interaction of genes for adaxial–abaxial polarity specification. Solid lines indicate direct regulation and dashed lines indicate indirect regulation or unconfirmed interactions. Faded names of genes indicate those to be repressed.

the abaxial-specific *ARF* gene family, *ETT/ARF3* and *ARF4*. These repression systems are experimentally demonstrated to be critical for development of the adaxial domain in *Arabidopsis* leaves. (2) The repression is further achieved by at least one other molecular system controlled by a modifier gene independently from the AS1–AS2 system. Although molecular mechanisms of the synergistic repression by AS1–AS2 and the modifiers have not been elucidated, their concerted actions should play a critical role in adaxial development. Events that repress the expression of these abaxial genes might occur in the presumptive adaxial domain of the leaf anlagen at its early developmental stages (p0–p1: Figure 7). Unlike the situation in maize, the contribution of tasiR-ARFs to adaxialization is not apparent in *Arabidopsis*. In the presumptive abaxial domain at such early stages, *AS2* is also directly repressed by KAN1 and KAN2 to induce abaxial specification.[33] Considered collectively, AS1–AS2 is a central player in the antagonistic interactions of genes expressed in the process of adaxial–abaxial polarity specification.

Recently, Qi et al.[108] have reported the existence of a transient low auxin zone in the adaxial domain of leaf primordia from p1 to p9, and suggested that auxin flow from leaf primordia to the SAM is responsible for the adaxial low auxin domain and, thus, acts as a signal influencing formation or maintenance of the leaf adaxial domain. The relationship between the auxin flow and the AS1-AS2-ETT pathway remains to be elucidated.

Antagonistic interaction has been proposed between *KAN* and *YAB* families and the *HD-ZIPIII* family in leaf polarity development. Recently, many phytohormone-related genes have been identified as candidates downstream of the respective *KAN1* and *REVOLUTA*, a member of the *HD-ZIPIII* family,[109–111] suggesting the involvement of genes for phytohormone biosynthesis, response, and transport in the antagonistic interaction. Molecular networks of the interaction are, however, still not clear. Loss-of-function *as2* mutations and double mutations of *AS2* and its modifier genes do not significantly affect expression of *HD-ZIPIII* genes,[32,70,90] suggesting that the adaxialization mediated by *HD-ZIPIII* is independent from that by AS1–AS2.

AS1–AS2 and various modifiers synergistically repress these developmentally important genes through certain nuclear processes. Taken together with these observations, it is likely that the repression system mediated by AS1–AS2 and modifier proteins might be, at least in part, involved in epigenetic processes. It will be intriguing to elucidate how coordinate actions of these molecules might determine epigenetic states of repression of *KNOX* and *ARF* family genes and how the *MET1*-dependent *ETT* methylation might be involved in establishment of the epigenetic state during leaf polarity specification.

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