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Note

ASYMMETRIC LEAVES2 and *FASCIATA2* cooperatively regulate the formation of leaf adaxial-abaxial polarity in *Arabidopsis thaliana*

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Abstract The establishment of adaxial–abaxial polarity in the early stage of leaf development is important for the expansion of lamina. In *Arabidopsis thaliana*, *asymmetric leaves2* (*as2*) and *as1* mutations cause defects in the leaf adaxial–abaxial polarity, which are exhibited as abaxialized filamentous leaves in the various modifier mutant backgrounds. Several modifier single mutants generate pointed leaves in common. Mutations in *FASCIATA2* (*FAS2*) also cause pointed leaves. The *FAS2* gene encodes a component of Chromatin Assembly Factor-1 (CAF-1), a histone chaperone, which affects mRNA levels of various genes through the regulation of chromatin states. In the present study, we demonstrate that *as2 fas2* double mutants frequently generate abaxialized filamentous leaves and show increased mRNA levels of genes for leaf abaxialization, *KANADI1* (*KAN1*), *KAN2*, *YABBY5* (*YAB5*), *ETTIN/AUXIN RESPONSE FACTOR3* (*ETT*/*ARF3*) and *ARF4*. In addition, the transcript levels of all four class 1 *KNOTTED1-like* homeobox genes that are involved in the maintenance of shoot apical meristem and the *Kip-related Protein2* (*KRP2*) and *KRP5* genes that are involved in the cell cycle progression are elevated in the *as2 fas2*. The mRNA levels of all genes other than *YAB5*, whose transcript levels rise in *as2 fas2*, were increased in the *fas2* single mutants. Our data suggest that FAS2 participates in the establishment of leaf adaxial–abaxial polarity through the repression of the transcript levels of these genes on the *as2* background.

Key words: *AS2*, *FAS2*, leaf development, adaxial–abaxial polarity, *Arabidopsis thaliana*.

In seed plants, aerial organs, such as leaves and floral organs, are initiated from the peripheral regions of the shoot apical meristem (SAM) composed of undifferentiated cells. Leaf primordia that are developed from the SAM grow up along the proximal-distal, media-lateral, and adaxial–abaxial axes through repeated cell divisions and cell differentiations. The establishment of adaxial–abaxial polarity at the initial stage of leaf development is crucial for the acquisition of leaf flatness (Steeves and Sussex 1989; Waites and Hudson 1995). *ASYMMETRIC LEAVES2* (*AS2*) and *AS1* genes of *Arabidopsis thaliana* function in the formation of appropriately expanded flat symmetric leaves, with generation of the three structural axes (Byrne et al. 2000 2001, 2002; Iwakawa et al. 2002; Lin et al. 2003; Ori et al. 2000; Rédei and Hirono 1964; Semiarti et al. 2001; Tsukaya and Uchimiya 1997; Xu et al. 2003). To date, various modifier mutations that generate filamentous leaves surrounded by abaxialized epidermis in the *as2* or *as1* mutant backgrounds have been identified [for example, *enhancer of asymmetric leaves2 and asymmetric leaves1-1* (*eal-1*) and *elongator3-27* (*elo3-27*), and so on], and it has been reported that the development of the adaxial domain of leaves is severely defective in these double mutants (Garcia et al. 2006; Horiguchi et al. 2011 a, b; Huang et al. 2006; Inagaki et al. 2009; Ishibashi et al. 2012; Kojima et al. 2011; Li et al. 2005; Pinon et al. 2008; Szakonyi and Byrne 2011; Ueno et al. 2007; Wang et al. 2011; Xu et al. 2012; Yang et al. 2006; Yao et al. 2008; Yuan et al. 2010). On the base of the extreme synthetic phenotype of the double mutants, it has been proposed that *AS2* (and *AS1*) and modifier genes are involved in adaxial development in independent and parallel fashions during the leaf formation. Interestingly, most of the single modifier mutants show the common phenotype, formation of slightly narrow leaves with pointed tip (pointed leaves). (Horiguchi et al. 2011; Ishibashi et al. 2012; Moschopoulos et al. 2012; Pinon et al. 2008). Mutations in the *FASCIATA2* (*FAS2*) gene, which encodes a component of Chromatin Assembly Factor-1 (CAF-1) complex, a histone chaperone, also generate such a leaf phenotype (Kaya et al. 2001). In the present paper, we examined whether the *fas2* mutation might also affect the establishment of leaf

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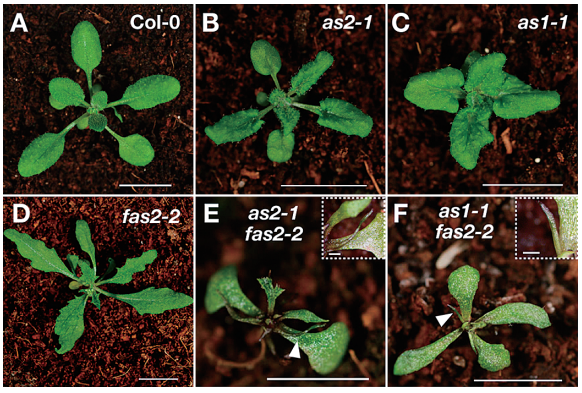

Figure 1. The *as2-1 fas2-2* and *as1-1 fas2-2* double mutants exhibited filamentous leaves. Gross morphology at 21 days after germination (A) Wild-type Col-0, (B) *as2-1*, (C) *as1-1*, (D) *fas2-2*, (E) *as2-1 fas2-2*, and (F) *as1-1 fas2-2* plants. Arrowheads indicate filamentous leaves. Higher-magnification views of filamentous leaves are shown in E and F. Bars: 10 mm (A–D), 5 mm (E, F), 0.5 mm in magnified views in E and F.

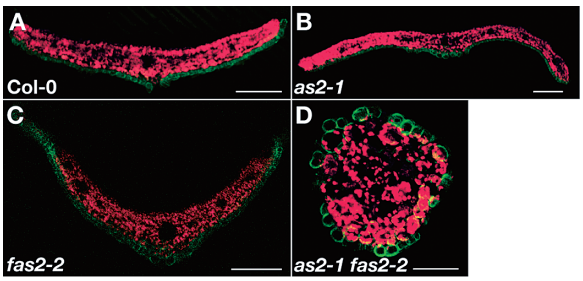

Figure 2. Filamentous leaves are abaxialized. Expression patterns of *FIL:GFP* in transverse sections of developing leaves at 16 days after germination. (A) Wild-type Col-0, (B) *as2-1*, (C) *fas2-2*, and (D) *as2-1 fas2-2* plants. Green, signals due to GFP; red, autofluorescence. Bars: 100 μm (A–C), 50 μm (D).

Table 1. Frequencies of plants with filamentous leaves.

Genotype	Number of plants examined	Percentage of plants with filamentous leaves
Col-0	96	0 (0)
as2-1	82	0 (0)
fas2-2	104	0 (0)
as2-1 fas2-2	136	95 (129)
as1-1	92	0 (0)
as1-1 fas2-2	71	25 (18)

Frequency is defined as the ratio of the number of plants with more than one filamentous leaf to the total number of plants examined. The numbers of plants with filamentous leaves are indicated in parentheses. Plants were grown at 22°C.

adaxial–abaxial polarity in *as2* mutant backgrounds and performed gene expression analyses in the *as2 fas2* double mutant. Xu et al. (2012) reported that mutation in *FASCIATA1* (*FAS1*) gene, which encodes another component of CAF-1, showed filamentous leaves in *as1* and *as2* mutant backgrounds, but histological and molecular characterizations behind the abnormal phenotypes remain to be determined. In addition, the genetic interaction between *as2* and *fas2* has not been investigated.

In this study, we used *fas2-2* loss-of-function mutants, which was originally isolated from ecotype Nossen. The *fas2-2* mutation causes fascinated stems, abnormal leaf phyllotaxy, narrow leaves and short roots (Kaya et al. 2001). As shown in Figure 1A–D and Table 1, although the wild-type Col-0, *as1-1*, *as2-1*, and *fas2-2* three-times-backcrossed with Col-0 plants did not have filamentous leaves, 25% and 95% of the *as1-1 fas2-2* and *as2-1 fas2-2* double mutants showed filamentous leaves, respectively. These results show that the *fas2-2* mutation influences the establishment of leaf adaxial–abaxial polarity in the *as1-1* and *as2-1* mutant backgrounds. The *as2-1 fas2-2* double mutant generated filamentous leaves with higher efficiency.

We investigated patterns of expression of cDNA for green fluorescent protein (GFP) under the control of the *FIL* promoter (*FILp:GFP*), which is expressed only in abaxial cells of leaf primordia (Ishibashi et al. 2012; Watanabe and Okada 2003). We observed signals due to GFP from *FILp:GFP* by using a confocal laser scanning microscope (LSM510 META; Carl Zeiss, Inc., Oberkochen, Germany). In the wild type, *as2-1*, and *fas2-2* plants, we detected GFP signals only in cells of the abaxial domain of expanded leaves (Figure 2A–C). In the filamentous leaves of *as2-1 fas2-2* double mutants, however, we detected GFP signals all over the epidermal cells (Figure 2D). These results suggest that the filamentous leaves in the *as2-1 fas2-2* double mutants are abaxialized.

By quantitative reverse transcriptional-PCR (qRT-PCR) using RNA from the shoot apices of wild-type, *as2-1*, *fas2-2*, and *as2-1 fas2-2* plants, we investigated the transcript levels of the genes that are involved in the establishment of leaf adaxial–abaxial polarity (Figure 3). We quantified transcripts of genes in the HD-ZIP III family [*PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), and *REVOLUTA* (*REV*)], which specify the adaxial cell fate; genes in the *KANADI* (*KAN*) family (*KAN1, KAN2*); genes in the *YABBY* (*YAB*) family [*FILAMENTOUS FLOWER* (*FIL*), *YAB2, YAB5*]; and genes in the *AUXIN RESPONSE FACTOR* (*ARF*) family [*ETTIN* (*ETT*) / *ARF3*, *ARF4*], which specify the abaxial cell fate, as described by Kojima et al. (2011). There were no significant differences among the mRNA levels of *PHB*, *PHV*, and *REV* in the wild-type, *as2-1*, *fas2-2*, and *as2-1 fas2-2* plants (Figure 3A). In contrast, the mRNA levels of *YAB5* and *ETT*/*ARF3* were increased in the *as2-1* mutant compared with those in the wild-type plants, consistent with our previous report (Figure 3B, Iwakawa et al. 2007). The mRNA levels of *KAN1*, *KAN2*, *ETT*/*ARF3*, and *ARF4* genes were increased in the *fas2-2* mutant compared with those in the wild-type plants (Figure 3B). In the *as2-1 fas2-2* double mutants, the mRNA levels of the genes, whose mRNA levels were up-regulated

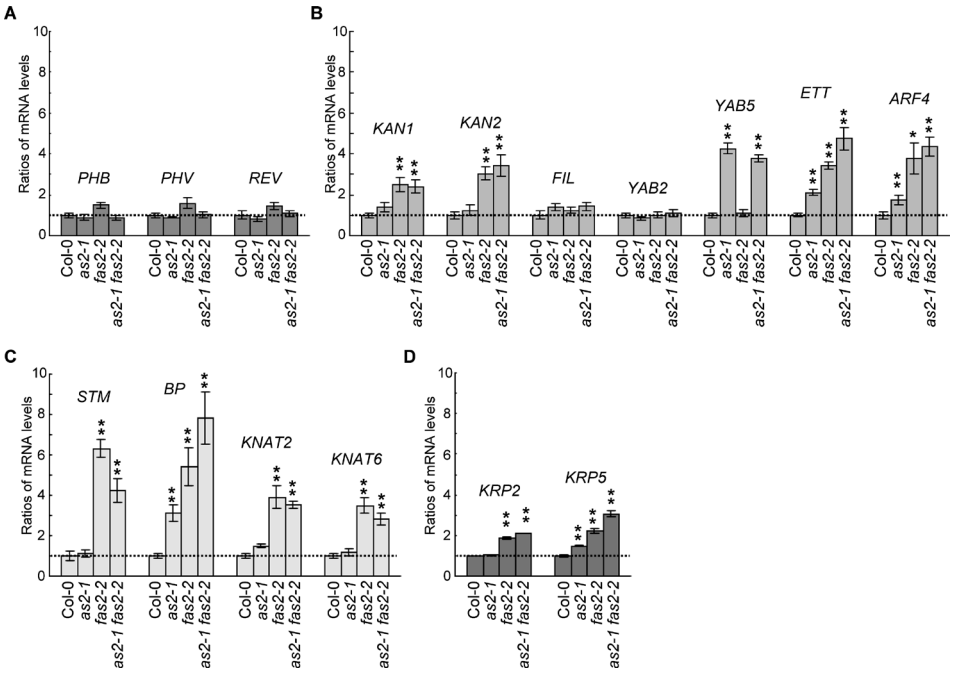

Figure 3. Expression analysis by qRT-PCR. Levels of relative expression of (A) genes involved in leaf adaxialization (HD-ZIPIII homeotic genes), (B) genes involved in leaf abaxialization (*KANADI*, *YABBY*, and *ARF* genes), (C) class1 *KNOX* genes, and (D) *KRP* genes, respectively, to those levels in the wild-type (Col-0) plants. Total RNA was extracted from shoot apices of 14-day-old Col-0, *as2-1*, *fas2-2*, and *as2-1 fas2-2*. Each value was normalized by reference to the level of *ACTIN2* (*ACT2*, At3g18780) transcripts. The values from wild-type plants were arbitrarily set as 1.0. Bars indicate the s.d. among more than three biological replicates. Significant differences from wild type were evaluated by Student's *t*-test and are represented by asterisks (* $p<0.05$ and ** $p<0.01$).

in either *as2-1* or *fas2-2*, increased, as compared with those in the wild type (Figure 3B). The mRNA level of *ETT*/*ARF3* in the *as2-1 fas2-2* double mutant was higher than that in both the *as2-1* and *fas2-2* single mutants, and the level of *ARF4* transcripts was higher than that in the *as2-1* single mutant (Figure 3B). These results are consistent with a conclusion that the filamentous leaves in the *as2-1 fas2-2* double mutants were abaxialized. To date, it has been reported that the formation of filamentous leaves in several double mutants combined with the *as2-1* mutant are, at least partially, due to increased mRNA levels of *ETT* and *ARF4* (Ishibashi et al. 2012; Takahashi et al. 2013). The increased levels of *ETT* and *ARF4* transcripts in the *as2-1 fas2-2* double mutant might be responsible for the formation of the filamentous leaves.

In the double mutants combined with the *as1* or *as2* mutation exhibiting the formation of filamentous leaves, the mRNA levels of class 1 *KNOTTED1-like* homeobox (*KNOX*) genes, *BREVIPEDICELLUS* (*BP*), *KNAT2*, *KNAT6*, and *SHOOT MERISTEMLESS* (*STM*), significantly increased, as compared with those of the wild type (Horiguchi et al. 2011a; Ishibashi et al. 2012; Kojima et al. 2011; Yang et al. 2006). We quantified the mRNA levels of these four class 1 *KNOX* genes in the wild-type, *as2-1*, *fas2-2*, and *as2-1 fas2-2* mutant plants (Figure 3C). Consistent with the previous reports, the mRNA levels of *BP* were raised in *as2-1* (Iwakawa et al. 2007; Semiarti et al. 2001). In *fas2-2*, the mRNA levels of all four class 1 *KNOX* genes were markedly increased over those levels in the wild type. In the *as2-1 fas2-2* double mutants, the mRNA levels of *KNAT2* and *KNAT6* were similar to those in the *fas2-2* single mutants, while the mRNA level of *STM* was higher than that of wild type, but lower than that of *fas2-2*. The level of *BP* transcripts accumulated in *as2-1 fas2-2* was further increased over those in the *fas2-2* mutant (Figure 3C). Ikezaki et al. (2010) have reported that the increased mRNA levels of *BP*, *KNAT2*, and *KNAT6* affect the leaf expansion along the proximal-distal direction, but they have no effect on the establishment of the leaf adaxial–abaxial polarity. However, the effects of elevated levels of *STM* mRNA on polarity have not been examined. A question remains whether *STM* might be involved in the establishment of the adaxial–abaxial polarity. The high efficiency of filamentous leaf formation that we observed might be due to the combined effects of the increased expression of all of *KAN1*, *KAN2*, *YAB5*, *ETT*/*ARF3*, *ARF4*, and all four class 1 *KNOX* genes.

In this report, we show a novel mutant with pointed leaves generated filamentous leaves in the *as1* and *as2* mutant backgrounds (Figure 1, Table 1). Takahashi et al. (2013) show that the mRNA levels of genes for the CDK inhibitor, Kip-related Proteins (KRPs), are increased, in common, in the *as2 eal* and *as2 elo3* double mutants exhibiting abaxialized filamentous leaves, and that the

increased levels of *KRP* transcripts are due to the up-regulation of *ETT*/*ARF3* in the *as2 eal* double mutant. We investigated the mRNA levels of *KRP* genes in the *as2-1 fas2-2* double mutants. In the *fas2-2* single mutant, the mRNA levels of *KRP2* and *KRP5* genes were increased by 1.9-fold and 2.2-fold, respectively, as compared to those of wild type (Figure 3D). The mRNA level of *KRP2* in *as2-1 fas2-2* was similar to that of *fas2-2*, that is, increased 2.0-fold over that of wild type. While, the mRNA level of *KRP5* in *as2-1 fas2-2* was increased by 3.1-fold over that of wild type, and was higher than that of *fas2-2* (Figure 3D). In *Arabidopsis thaliana*, the overexpression of both *KRP2* and *KRP5* stimulate the transition from the mitotic cell cycle to the endoreduplication cycle (Jégu et al. 2013; Verkest et al. 2005; Wen et al. 2013), suggesting that these *KRP* genes have a negative effect on cell cycle progression. The formation of filamentous leaves in these double mutants might be caused by inhibition of cell division for the lateral expansion of leaves due to the misexpression of *KRP* genes. In the wild type, precise modulation of *KRPs* expression might be critical for the development of flat leaves. Adachi et al. (2009) reported that the *CDKA;1* promoter region contains a regulatory region that controls abaxial side-biased expression. Our microarray analyses, however, showed that levels of *CDKA;1* transcripts were not significantly increased in shoot apices of mutant plants forming abaxialized filamentous leaves (our unpublished data). The abaxial region-biased expression of CDKA;1 may reflect differential cell division activity on each side of leaves; thus, KRP misexpression in double mutants might inhibit CDK activity more on the adaxial region.

The class 1 *KNOXs, ETT, ARF4, KAN1, KAN2, KRP2*, and *KRP5* were all increased in the *fas2-2* mutant, suggesting that FAS2 represses the levels of these gene transcripts, although it remains unclear whether the effects of FAS2 is direct or indirect. The AS1–AS2 complex (Guo et al. 2008; Yang et al. 2008) directly repressed transcript levels of *BP, KNAT2*, and *ETT*/*ARF3* (Guo et al. 2008; Iwasaki et al. 2013). FAS2 is an *Arabidopsis* ortholog of a p60 subunit of the CAF-1 complex, which has activity of histone chaperone and affects the mRNA levels of various genes including those involved in the cell cycle progression (Kaya et al. 2001; Ono et al. 2006; Schönrock et al. 2006). It should be intriguing to examine whether the genes whose transcript levels are increased in *fas2-2*, as described above, are responsible for the morphological phenotypes.

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