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Original Paper

A genetic link between epigenetic repressor AS1–AS2 and DNA replication factors in establishment of adaxial–abaxial leaf polarity of *Arabidopsis*

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Abstract Balanced development of adaxial and abaxial domains in leaf primordia is critical for the formation of flat symmetric leaf lamina. Arabidopsis ASYMMETRIC LEAVES1 (AS1) and AS2 proteins form a complex (AS1–AS2), which acts as key regulators for the adaxial development by the direct repression of expression of the abaxial gene *ETTIN*/*AUXIN RESPONSE FACTOR3* (*ETT*/*ARF3*). Many modifier mutations have been identified, which enhance the defect of *as1* and *as2* mutations to generate abaxialized filamentous leaves without adaxial traits, suggesting that the development of the adaxial domain is achieved by cooperative repression by AS1–AS2 and the wild-type proteins corresponding to the modifiers. Mutations of several genes for DNA replication-related chromatin remodeling factors such as Chromatin Assembly Factor-1 (CAF-1) have been also identified as modifiers. It is still unknown, however, whether mutations in genes involved in DNA replication themselves might act as modifiers. Here we report that *as1* and *as2* mutants grown in the presence of hydroxyurea, a known inhibitor of DNA replication, form abaxialized filamentous leaves in a concentration-dependent manner. We further show that a mutation of the *INCURVATA2* (*ICU2*) gene, which encodes the putative catalytic subunit of DNA polymerase α, and a mutation of the *Replication Factor C Subunit3* (*RFC3*) gene, which encodes a protein used in replication as a clamp loader, act as modifiers. In addition, *as2-1 icu2-1* double mutants showed increased mRNA levels of the genes for leaf abaxialization. These results suggest a tight link between DNA replication and the function of AS1–AS2 in the development of flat leaves.

Key words: *ASYMMETRIC LEAVES2*, DNA replication, *ICU2*, leaf development, *RFC3*.

Introduction

Leaf primordia that are developed as lateral organs from the shoot apical meristem (SAM) grow up along the proximal–distal, medial–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 formation of flat symmetric leaves (Bowman and Floyd 2008; Byrne et al. 2000; Hudson 2000; Nakata and Okada 2013; Semiarti et al. 2001; Steeves and Sussex 1989; Szakonyi and Byrne 2011; Tsukaya 2013; Waites and Hudson 1995). The abaxialization is thought to proceed to adaxialization because abaxial-determining genes were expressed earlier than that of adaxial-determining genes (Eshed et al. 1999, 2001; Sawa et al. 1999) and the defect of the adaxial domains results in the generation of the filamentous leaf that retains only abaxial traits (Li et al. 2005; Ueno et al. 2007; Waites and Hudson 1995). *AS1* and *AS2* are key regulators of the formation of flat symmetric leaves. *AS1* and *AS2* encode nuclear proteins and form a complex (designated AS1–AS2) (Guo et al. 2008; Luo et al. 2012; Xu et al. 2003; Yang et al. 2008). Mutations in these genes are associated with pleiotropic abnormalities in leaves along the three developmental axes described above (Byrne et al. 2000; Iwakawa et al. 2002, 2007; Matsumura et al. 2009; Ori et al. 2000; Rédei and Hirono 1964; Semiarti et al. 2001; Tsukaya and Uchimiya 1997), suggesting that AS1–AS2 regulates multiple genes (Iwasaki et al. 2013; Machida et al. 2015; Takahashi et al. 2008; Takahashi et al. 2013) that might be involved in leaf formation along the three axes.

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Direct repression by AS1–AS2 of the expression of at least two gene families is critical for leaf development, one of which is the class 1 KNOTTED-like homeobox (*KNOX*) genes such as *BREVIPEDICELLUS* (*BP*), *KNAT2* (Guo et al. 2008). The other class of direct targets includes the abaxial-determining gene *ETTIN*/*AUXIN RESPONSE FACTOR3* (*ETT*/*ARF3*) (Iwasaki et al. 2013), which is directly repressed by the binding of AS1–AS2 to its promoter region. In addition, AS1–AS2 also indirectly represses the expression of *ETT*/*ARF3* and the functionally redundant gene *ARF4* through its positive regulation of the miR390-tasiR-ARF pathway, essential for adaxial development (Iwasaki et al. 2013). AS1–AS2 is also required for maintaining levels of methylated CpGs in the *ETT*/*ARF3* coding region (Iwasaki et al. 2013). The methylation is abolished in the mutant of *METHYLTRANSFERASE1* (*MET1*) gene and the level of *ETT*/*ARF3* transcript increases in shoot apices in *met1* (Iwasaki et al. 2013). The observed anti-parallel relationship supports the hypothesis that the CpG methylation in *ETT*/*ARF3* might play a role in repression of *ETT*/*ARF3* expression (Iwasaki et al. 2013).

Defects in polarity of *as1* and *as2* leaves are enhanced under certain growth conditions as well as in conjunction with mutations in members of certain groups of genes (Machida et al. 2015), which are designated as modifiers of adaxial–abaxial patterning (Machida et al. 2015; Matsumura et al. 2016; Szakonyi et al. 2010). To date, various modifier mutations that generate filamentous leaves surrounded by abaxialized epidermis in the *as1* or *as2* mutant backgrounds have been identified and it has been reported that the development of the adaxial domain of leaves is severely defective in those double mutants. The modifier genes include several that mediate the biogenesis of tasiR-ARF (Machida et al. 2015; Yang et al. 2006). Other relevant modifier genes belong to several different groups: those for ribosome biogenesis (Horiguchi et al. 2011a; Matsumura et al. 2016; Pinon et al. 2008; Yao et al. 2008); chromatin modification (Kojima et al. 2011; Ueno et al. 2007); and cell proliferation (Horiguchi et al. 2011b; Ishibashi et al. 2012; Wang et al. 2011; Xu et al. 2012; Yuan et al. 2010). Mutations of genes related to DNA replication-related chromatin remodeling and DNA repair have been identified as modifiers that enhance leaf adaxial–abaxial abnormalities in *as1* and *as2* (Inagaki et al. 2009; Machida et al. 2015; Xu et al. 2012). Mutations in *FASCIATA1* (*FAS1*) and *FAS2* genes that encode *Arabidopsis* homologs of components of Chromatin Assembly Factor-1 (CAF-1), a histone chaperone essential for chromatin remodeling after DNA replication (Takami et al. 2007), also cause abaxialized filamentous leaves in *as1* and *as2* (Ishibashi et al. 2013; Xu et al. 2012). Since these factors are not directly involved in reactions of DNA replication, the relationship between AS1–AS2, factors involved in DNA replication and the leaf development is, however, still unknown.

In the present study, we used hydroxyurea, which is an inhibitor of DNA replication (Saban and Bujak 2009), and *Arabidopsis* mutants of genes for the catalytic subunit of DNA polymerase α and Replication Factor C Subunit3 (RFC3) involved in DNA elongation on a primed DNA template as a clamp loader. The present results show that *as1* and *as2* mutants grown in the hydroxyurea-containing medium formed abaxialized filamentous leaves in a concentration-dependent manner. The mutations we used also generated abaxialized filamentous leaves in the *as1* and *as2* backgrounds. These results suggest that the cooperative action of AS1–AS2 and the proper progression of DNA replication are involved in the development of flat leaves in *Arabidopsis*.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Col-0 (CS1092), and the mutants *as1-1* (CS3374) and *as2-1* (CS3117) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA; ABRC). We outcrossed *as2-1* with Col-0 three times and *as1-1* with Col-0 once, and used the progeny for our experiments (Semiarti et al. 2001). The *incurvata2-1* (*icu2-1*) mutant that has a point mutation in the coding region of *INCURVATA2*, which encodes the putative catalytic subunit of the DNA polymerase α of *Arabidopsis thaliana*, was described by Barrero et al. (2007). The *rfc3-1* mutant, which has a point mutation in the coding region of a gene encoding a protein with high homology to Replication Factor C Subunit3 of yeast and other eukaryotes, was described by Xia et al. (2009). For analysis of phenotypes, seeds were sown on soil or on agar plates of Murashige and Skoog (MS) medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 2% (w/v) sucrose and 0.8% agar (Nakagawa et al. 2012). After 2 days at 4°C in darkness, plants were transferred to a daily regimen of 8 h of darkness and 16 h of white light at 50 μmol m^{-2} s^{-1} at 22°C, as described previously (Semiarti et al. 2001). Hydroxyurea was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). For chemical treatments, hydroxyurea was dissolved in H_2O, mixed with MS agar, and immediately dispensed into plastic dishes. Ages of plants are given in terms of numbers of days after sowing.

Fluorescence microscopy

The *as1-1* and *as2-1* plants containing the *FILAMENTOUS FLOWER* (*FIL*) promoter *FILp:GFP* were described by Nakagawa et al. (2012). Shoot apices containing leaf primordia were embedded in 5% agar and then the agarose blocks were sliced into sections with a vibratome. Fluorescence was observed with a confocal laser scanning microscope (LSM510 META; Carl Zeiss Inc., Oberkochen, Germany).

Real-time RT-PCR

Shoot apices of mutant and Col-0 (wild-type) plants were harvested 14 days after seeds were sown and immediately frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated from the 14-day-old shoot apices with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. For the analysis of RNA levels in Arabidopsis by real-time qRT-PCR, we prepared 10 μg of total RNA. Reverse transcription was carried out with ReverTra Ace (TOYOBO, Osaka, Japan). Sample volumes were normalized for equal amplification of DNA fragments using primers specific for α-tubulin cDNA. Primer sets are listed in the Supplementary Table. PCR was performed in the presence of the double-stranded DNA-specific dye SYBR Green (Applied Biosystems, Lincoln, CA, USA). Amplification was monitored in real time with the Applied Biosystems StepOnePlus Real-Time PCR system (Applied Biosystems) according to the supplier's recommendations. The mean value of three technical replicates was normalized by using the *ACTIN2* transcripts as a control.

Results

Treatments of as1 and as2 plants with hydroxyurea induce filamentous leaves

Col-0 (wild-type), *as1-1* and *as2-1* plants were grown in the presence of hydroxyurea, a known replication inhibitory compound. Using 0 to 10 mM concentrations of hydroxyurea, we examined the dose-dependent effects of this compound on the formation of expanded true leaves and filamentous leaves in these plants (Figure 1A–C). The percentage of plants with expanded true leaves was decreased at higher concentrations of hydroxyurea in *as1-1*, *as2-1*, and the wild type (Figure 1B). In the presence of 3 mM hydroxyurea, Col-0, *as1-1* and *as2-1* plants normally formed expanded true leaves. Mutants, however, exhibited higher sensitivity to hydroxyurea than Col-0 between 4 and 6 mM: all Col-0 and *as1-1* plants and 90% of *as2-1* formed expanded true leaves at 4 mM; 90% of the wild type and more than 60–75% of mutants formed expanded leaves at 5 mM; 72% of Col-0 and only 7% of mutants formed expanded leaves at 6 mM. As shown in Figure 1C, 8%, 26%, and 34% of *as1-1* and 19%, 38%, and 46% of *as2-1* plants produced filamentous leaves indicative of a defect in adaxial–abaxial polarity at 3, 4, and 5 mM hydroxyurea, respectively. In contrast, only 1% and 9% of Col-0 plants with filamentous leaves were observed at 4 and 5 mM concentrations of hydroxyurea, respectively. Thus, frequencies of the formation of filamentous leaves in *as1-1* and *as2-1* increased in a concentration-dependent manner by hydroxyurea. Most of all mutant plants stopped developing true leaves and growing in the presence of 6 mM hydroxyurea (Figure 1A, C), and they eventually exhibited chrolosis (Figure 1A). Seventy-two percent of Col-0 plants generated expanded leaves and 25% generated filamentous leaves under the same growth conditions (Figure 1B, C). Thus, a concentration of 6 mM hydroxyurea should be too strong to use for examining leaf phenotypes in *as1-1* and *as2-1* mutants, and a concentration of 5 mM or less would be suitable for the examination.

Since filamentous leaves are often generated in plants having defective adaxial–abaxial polarity determination, we examined whether the leaf phenotypes induced by the hydroxyurea treatment are associated with transcript levels of genes that are involved in the polarity development. We observed signals due to green fluorescent protein (GFP), synthesis of which is under the control of the promoter of the abaxial-determining gene *FILAMENTOUS FLOWER* (*FIL*) (*FILp:GFP*) (Watanabe and Okada, 2003) in transgenic *as1-1* and *as2-1* mutants. As shown in Figure 1D, strong signals were detected in cells located at peripheral positions in filamentous leaves of *as1-1* and *as2-1* plants grown in the presence of 4 mM hydroxyurea, whereas no signals were detected in cells located on the adaxial side of leaves of *as1-1* and *as2-1* plants in the absence of hydroxyurea. These results suggested that filamentous leaves in *as1-1* and *as2-1* treated with hydroxyurea were abaxialized.

Mutation of DNA polymerase α enhanced a leaf adaxial–abaxial polarity defect in as1 and as2

The results in the previous section suggest that inhibition of DNA replication preferentially interfere development of the adaxial domain during formation of leaf primordia on the background of *as1-1* and *as2-1*. We next examined whether the mutation (*icu2-1*) of the *INCURVATA2* gene, which encodes an *Arabidopsis* homolog of the catalytic subunit of the DNA polymerase α (Barrero et al. 2007), an essential gene for DNA replication, might affect adaxial–abaxial polarity establishment of leaves in the *as1* and *as2* mutant backgrounds. For this, we generated the double mutants *as1-1 icu2-1* and *as2-1 icu2-1*. As shown in Figure 2 and Table 1, 18.1–21.7% of *as1-1 icu2-1* and 4.6–11.7% of *as2-1 icu2-1* double mutants showed filamentous leaves, although the wild-type plant and any single mutants of *as1-1*, *as2-1*, and *icu2-1* did not generate filamentous leaves. We also observed trumpet-like leaves, the weak phenotype of filamentous leaves. Trumpet-like leaves were seen on 16.4–26.1% of *as1-1 icu2-1* and 3.0–5.5% of *as2-1 icu2-1*. These results suggested that the *icu2-1* mutation influenced the establishment of leaf adaxial–abaxial polarity in the *as1-1* and *as2-1* mutant backgrounds.

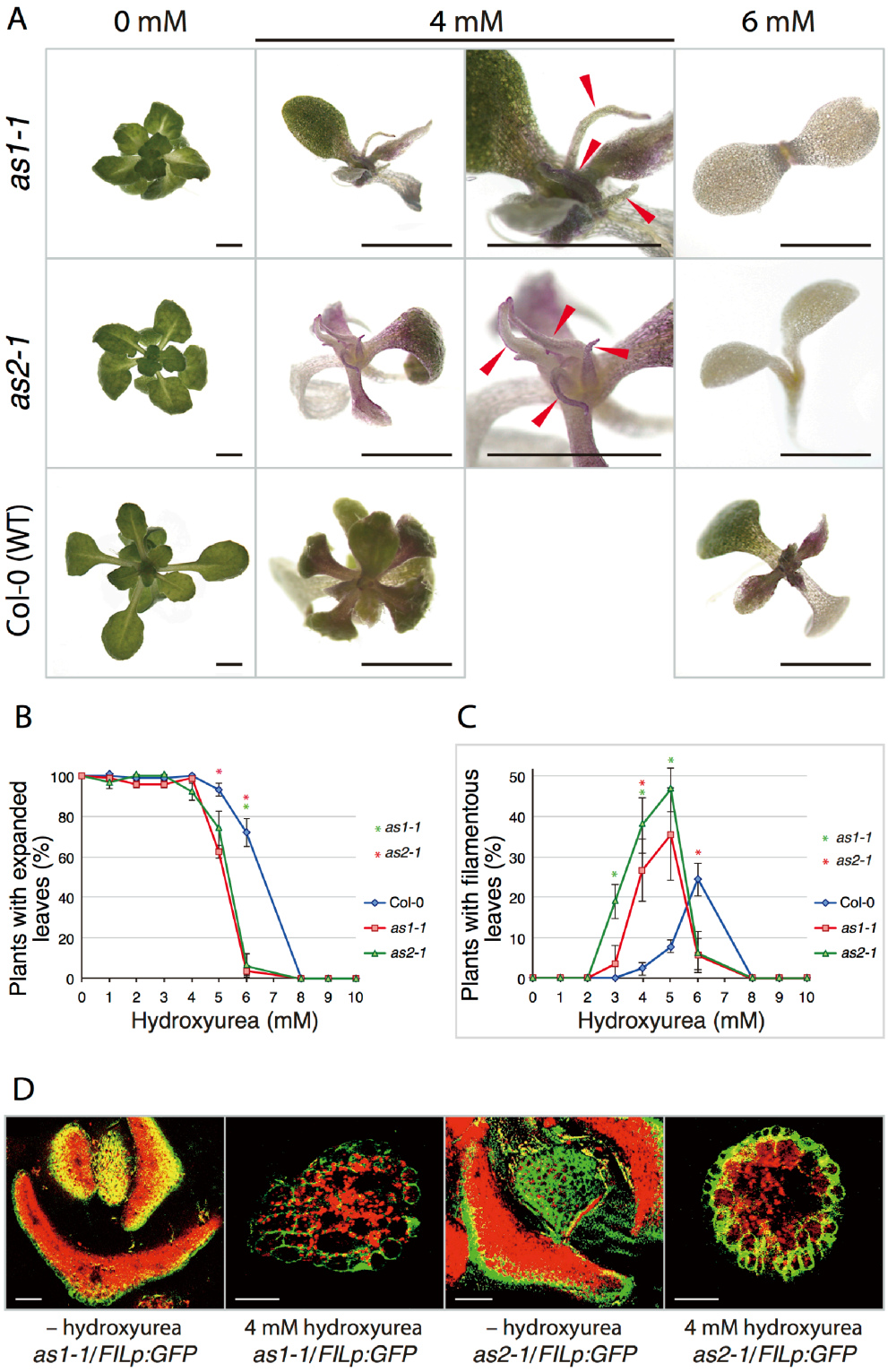

Figure 1. Effects of hydroxyurea on *as1-1* and *as2-1* mutants. (A) Phenotypes of Col-0, *as1-1*, and *as2-1* plants grown on agar plates in the presence and the absence of 4 mM, 6 mM hydroxyurea. Red arrowheads on *as1-1* and *as2-1* plants show filamentous leaves. Scale bars=2 mm. (B) Frequencies of plants that have true leaves grown in the presence and in the absence of hydroxyurea. Frequency is defined as the ratio of the number of plants having expanded leaves to the total number (n=30) of plants examined. Plants were grown at 22°C. Observations were performed at 21 days after sowing. Bars represent the s.d. from three biological replicates. (C) Frequencies of plants with filamentous leaves grown in the presence and in the absence of hydroxyurea. Frequency is defined as the ratio of the number of plants with more than one filamentous leaf to the total number (n=30) of plants examined. Plants were grown at 22°C. Observations were performed at 21 days after sowing. Bars represent the s.d. from three biological replicates. (D) *as1-1/FILp:GFP* and *as2-1/FILp:GFP* plants were grown on medium with and without 4 mM hydroxyurea for 21 days. Expression patterns of *FILp:GFP* in transverse sections of leaves are shown. Green signals due to GFP; red, autofluorescence. Yellow signals were due to both GFP and autofluorescence. Scale bars=50 μm. Significant differences from wild type were evaluated by Student's t-test and are represented by asterisks (* p<0.01) in (B) and (C).

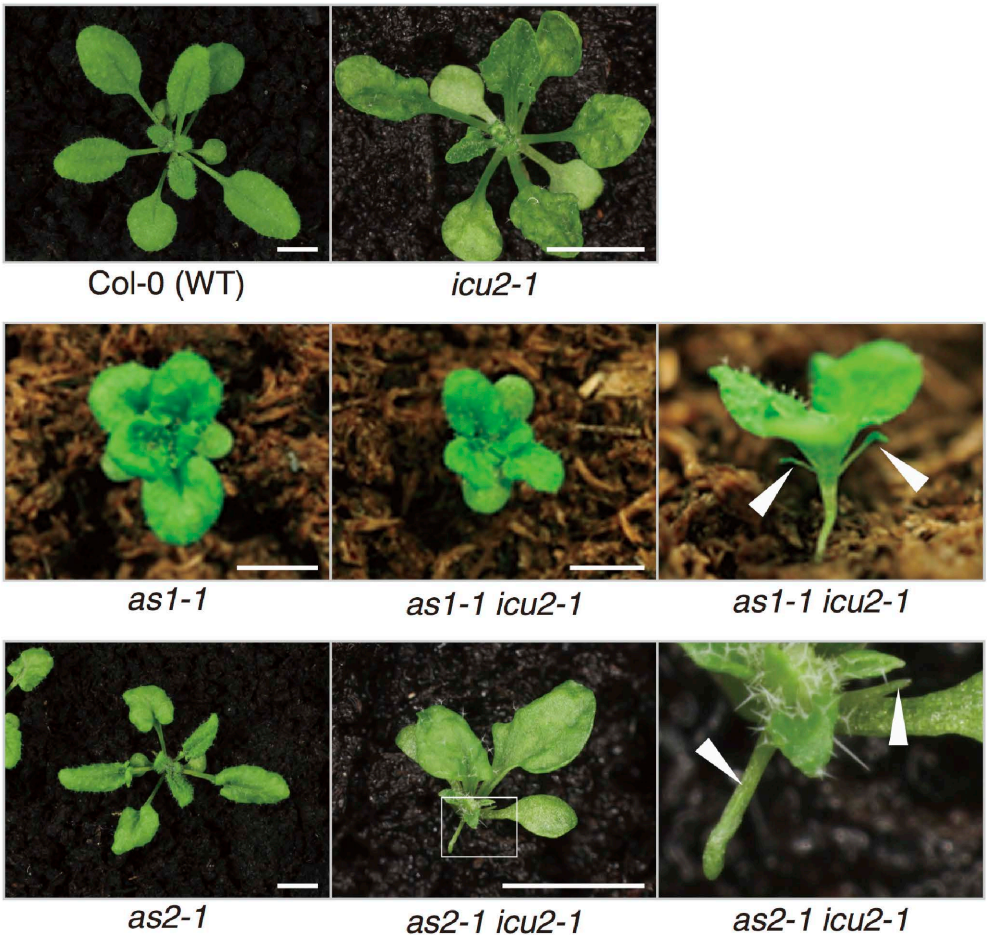

Figure 2. The *inc2-1* mutation enhanced the leaf-phenotype in *as1-1* and *as2-1* mutants. Gross morphology at 21 days after sowing. Plants indicated below pictures were grown as described in Materials and methods. The *as1-1 icu2-1* and *as2-1 icu2-1* double mutants exhibited filamentous leaves. Arrowheads indicate filamentous leaves. Scale bars = 5 mm.

Levels of transcripts of the abaxial determinant genes and class 1 KNOX genes were increased in as2-1 icu2-1

In the previous section, we have shown that the filamentous leaves induced by the hydroxyurea treatment were abaxialized (Figure 1D), suggesting that patterns of expression of genes that control abaxial–adaxial polarity establishment might be altered in the mutants we used. We performed real-time RT-PCR using RNA from the shoot apices of Col-0 (wild-type), *as2-1*, *icu2-1*, and *as2-1 icu2-1* plants. We quantified transcripts of *PHABULOSA* (*PHB*), one of the genes in the *HD-ZIP III* family that specify the adaxial cell fate; *ETT*/*ARF3*, *ARF4*, *KANADI2* (*KAN2*), *FIL*, *YABBY5* (*YAB5*) genes that specify the abaxial cell fate; and the *BP*, *KNAT2*, *SHOOT MERISTEMLESS* (*STM*) genes, which are members of the class 1 *KNOX* gene family and are expressed in the SAM and its periphery in wild-type plants. As shown in Figure 3, the transcript levels of *BP*, *KAN2*, *FIL* and *YAB5* genes were significantly increased in the *as2-1* mutant compared with those in Col-0 (wild-type) plant, consistent with our previous report (Kojima et al. 2011; Ishibashi et al. 2013). The transcript levels of *KNAT2*, *STM*, *ETT*/*ARF3*, *ARF4*, *KAN2* genes were increased in the *icu2-1* mutant compared with those in Col-0 (wild-type) plant. The transcript level of the *PHB* gene was decreased in the *as2-1*, *icu2-1* and *as2-1 icu2-1* plants. Furthermore, The accumulated transcript levels of either *ETT*/*ARF3* or *ARF4* in the *as2-1 icu2-1* double mutant were significantly higher than those levels in both the *as2-1* and *icu2-1* single mutants.

To date, it has been reported that the formation of filamentous and trumpet-like leaves in several double mutants combined with the *as2-1* mutant is, at least partially, due to the increased accumulation of levels of *ETT*/*ARF3* and *ARF4* transcripts. These results support the effects of the *icu2-1* mutation on the establishment of leaf adaxial–abaxial polarity in the *as1-1* and *as2-1* mutant background. The increased levels of *ETT*/*ARF3* and *ARF4* transcripts in the *as2-1 icu2-1* double mutant might be responsible for the formation of the filamentous and trumpet-like leaves.

In the double mutants combined with the *as2* mutation that exhibit the formation of filamentous

Table 1. Number of plants with trumpet-like leaves or filamentous leaves.

Genotype	Experiments	Number of plants examined	Number of plants with trumpet-like leaves (%)	Number of plants with filamentous leaves (%)
Col-0 (WT)	1	180	0 (0)	0 (0)
	2	165	0 (0)	0 (0)
	3	173	0 (0)	0 (0)
as1-1	1	113	0 (0)	0 (0)
	2	104	0 (0)	0 (0)
	3	97	0 (0)	0 (0)
as2-1	1	273	0 (0)	0 (0)
	2	193	0 (0)	0 (0)
	3	209	0 (0)	0 (0)
icu2-1	1	251	0 (0)	0 (0)
	2	186	0 (0)	0 (0)
	3	215	0 (0)	0 (0)
as1-1 icu2-1	1	120	23 (19.2)	21 (17.5)
	2	138	36 (26.1)	30 (21.7)
	3	116	19 (16.4)	21 (18.1)
as2-1 icu2-1 (C2)	1	255	11 (4.3)	19 (7.5)
	2	231	7 (3.0)	27 (11.7)
	3	241	10 (4.1)	24 (10.0)
as2-1 icu2-1 (E7)	1	227	8 (3.5)	18 (7.9)
	2	238	13 (5.5)	11 (4.6)

Number of plants with trumpet-like or filamentous leaves is defined as the number of plants with more than one trumpet-like or filamentous leaves, respectively. Percentages in parenthesis indicate the frequency with which trumpet-like or filamentous leaves structure was observed relative to the total number of plants examined. Plants were grown at 22°C for 21–23 days after sowing. C2 and E7 indicate different offspring lines generated by crossing *as2-1* with *icu2-1*.

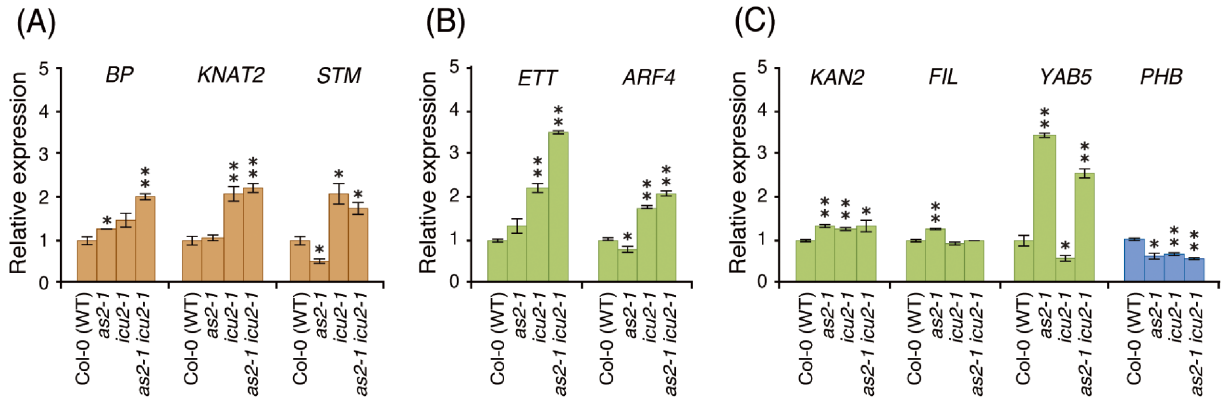

Figure 3. Transcript levels of genes involved in the determination of leaf polarity and class 1 *KNOX* genes. Levels of relative expression of (A) class 1 *KNOX* genes, (B) genes involved in leaf abaxialization (*ETT* and *ARF4*), (C) other genes that are involved in leaf abaxialization (*KAN2*, *FIL*, *YAB5*), and a gene that is involved in leaf adaxialization (*PHB*), respectively, relative 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*, *icu2-1*, and *as2-1 icu2-1*. Each value was normalized by reference to the level of *ACTIN2* (*ACT2*, at3g18780) transcripts. Light brown, light green and light blue show class 1 *KNOX* genes, abaxial determinant genes and an adaxial determinant gene, respectively. The values from wild-type plants were arbitrarily set at 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 ** $p<0.01$).

leaves, the accumulated transcript levels of the class 1 *KNOX* genes *BP*, *KNAT2*, *KNAT6*, and *STM* have been significantly increased, as compared with those of the wild type (Horiguchi et al. 2011b; Ishibashi et al. 2012; Kojima et al. 2011; Yang et al. 2006). As shown in Figure 3A, in *as2-1 icu2-1*double mutant, the mRNA levels of *BP*, *KNAT2* and *STM* genes were markedly increased over those levels in Col-0 (wild-type). These results suggested that leaves in *as2-1 icu2-1* double mutants were indeterminate and abaxialized.

Mutation of an Arabidopsis homolog of Replication Factor C subunit 3 (RFC3) enhanced a leaf adaxial–abaxial polarity defect in as1 and as2

To generalize the observation obtained in the above experiment with *as2-1 icu2-1*, we examined the *rfc3-1* mutant for the potential role in leaf development. It is a mutant allele of *AtRFC3*, which encodes an *Arabidopsis* homolog of Replication Factor C subunit 3 (Xia et al. 2009; 2010) and is potentially required for stable DNA replication by DNA polymerases (delta and epsilon) (Yin et al. 2009). Leaves of the

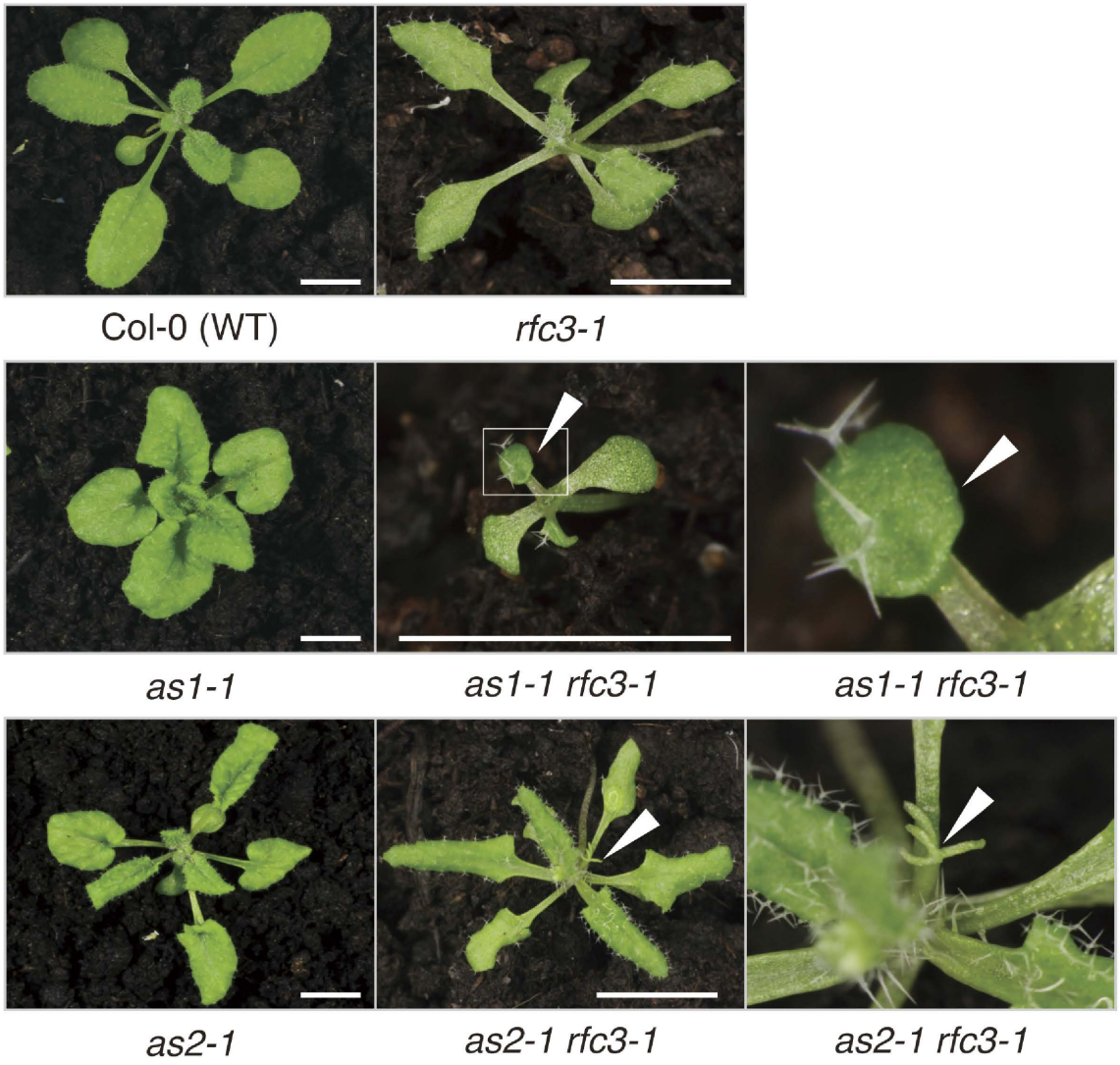

Figure 4. The *rfc3-1* mutation enhanced the leaf-phenotype in *as1-1* and *as2-1* mutants. The *as1-1 rfc3-1* double mutants exhibited trumpet-like leaves, while the *as2-1 rfc3-1* double mutants exhibited filamentous leaves. Gross morphology at 21 days after sowing. Arrowheads indicate higher magnification views of trumpet-like leaves or filamentous leaves. Scale bars = 5 mm.

rfc3-1 mutant were narrow and pointed (Figure 4), which is one of characteristic leaf abnormalities by the modifier mutations to enhance leaf polarity defects in *as1* and *as2* (Matsumura et al. 2016). To further confirm the involvement of *AtRFC3* in the leaf polarity development, we examined leaf phenotypes in the double mutants *as1-1 rfc3-1* and *as2-1 rfc3-1*. As shown in Figure 4 and Table 2, 4.7–7.8% and 11.1–14.5% of the *as1-1 rfc3-1* and *as2-1 rfc3-1* double mutants showed filamentous leaves, respectively. Trumpet-like leaves were seen on 5.9–8.2% of *as1-1 rfc3-1* and 0–2.6% of *as2-1 rfc3-1*. These results suggested that the *rfc3-1* mutation influenced in a certain extent the establishment of leaf adaxial–abaxial polarity in the *as1-1* and *as2-1* mutant backgrounds.

Discussion

The results in the present study showed that hydroxyurea, which interferes DNA synthesis, disrupted the establishment of the leaf adaxial–abaxial polarity in the *as1* or *as2* mutant, which resulted in the formation of the abaxialized filamentous leaves (Figure 1A, C, D). Hydroxyurea, a known replication inhibitory compound, inhibits ribonucleotide reductase enzyme known to be crucial in the conversion of ribonucleotides into deoxyribonucleotides and prevents cells from leaving the G1/ S phase of the cell cycle (Saban and Bujak 2009). Our results imply that normal progression of the replication step might be required for adaxial–abaxial establishment of leaf development. In addition, mutations of *ICU2* and *RFC3* genes that are required for DNA replication also induced the formation of filamentous leaves in the *as1* or *as2* genetic background (Figures 2 and 4). These

Table 2. Number of plants with trumpet-like leaves or filamentous leaves.

Genotype	Experiments	Number of plants examined	Number of plants with trumpet-like leaves	Number of plants with filamentous leaves
Col-0 (WT)	1	90	0 (0)	0 (0)
	2	78	0 (0)	0 (0)
	3	85	0 (0)	0 (0)
as1-1	1	93	0 (0)	0 (0)
	2	90	0 (0)	0 (0)
	3	71	0 (0)	0 (0)
as2-1	1	68	0 (0)	0 (0)
	2	76	0 (0)	0 (0)
	3	81	0 (0)	0 (0)
rfc3-1	1	86	0 (0)	0 (0)
	2	85	0 (0)	0 (0)
	3	80	0 (0)	0 (0)
as1-1 rfc3-1	1	102	7 (6.9)	8 (7.8)
	2	85	5 (5.9)	4 (4.7)
	3	85	7 (8.2)	6 (7.1)
as2-1 rfc3-1	1	63	0 (0)	8 (12.7)
	2	76	2 (2.6)	11 (14.5)
	3	81	1 (1.2)	9 (11.1)

Number of plants with trumpet-like or filamentous leaves is defined as the number of plants with more than one trumpet-like or filamentous leaves, respectively. Percentages in parenthesis indicate the frequency with which trumpet-like or filamentous leaves structure was observed relative to the total number of plants examined. Plants were grown at 22°C for 21–23 days after sowing.

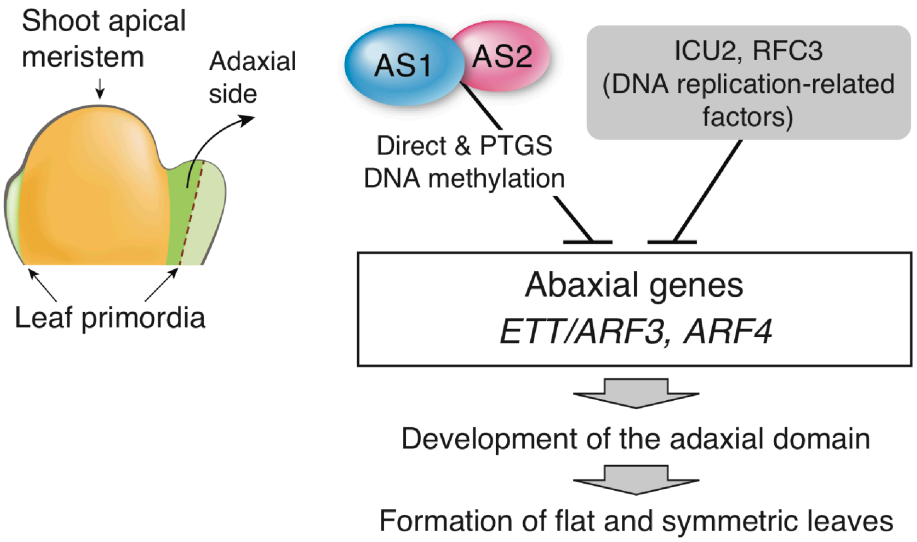

Figure 5. Roles of AS1–AS2, *ICU2*, and *RFC3* in leaf development in *A. thaliana*. The AS1–AS2 complex and DNA replication-related factors ICU2 (or RFC3) act cooperatively to repress expression of the leaf abaxial determinant gene *ETT*/*ARF3* and *ARF4*. Repression of *ARF*s is crucial for development of the adaxial domain of leaves and then formation of flat and symmetric leaves.

results suggested that a proper progression of DNA replication is critical, at least in part, for the development of the adaxial domain in *as1* and *as2* mutants. Since the frequencies of formation of filamentous leaves were increased in the *as1* or *as2* mutant treated with hydroxyurea or in the double mutants, *as1-1 icu2-1*, *as2-1 icu2-1*, *as1-1 rfc3-1*, *as2-1 rfc3-1*, the AS1–AS2-mediated pathway and the progression of DNA replication might be independently, but cooperatively, involved in the development of the adaxial domain of leaves in the wild-type Arabidopsis (Figure 5). Such cooperative actions in the leaf development are also observed in combinations of *as* mutations with other modifiers (Machida et al. 2015).

The transcript level of *ETT*/*ARF3* gene, which is a direct target of AS1–AS2 (Iwasaki et al. 2013), was increased in the *icu2-1* mutant. Furthermore, transcript levels of *ETT*/*ARF3* and *ARF4* in the *as2-1 icu2-1* double mutant were higher than those in both the *as2-1* and *icu2-1* single mutants (Figure 3). Since we have already shown that repression of expression of the abaxial genes *ETT*/*ARF3* and *ARF4* by AS1–AS2 and modifiers is important to form flat leaves (Iwasaki et al. 2013; Matsumura et al. 2016; Takahashi et al.

2013), phenotypes of the filamentous and trumpet-like leaves observed in *as1-1 icu2-1* and *as2-1 icu2-1* might be caused similarly by the up-regulation of *ETT*/*ARF3* during leaf development. It has been shown that *ICU2* genetically interacts with *TERMINAL FLOWER2*, which encodes an ortholog of HETEROCHROMATIN PROTEIN1 of animals and yeasts, and *CURLY LEAF*, the Polycomb group (PcG) gene (Barrero et al. 2007). Another study of the *icu2-1* mutant revealed that *ICU2* is required for ensuring the stable maintenance of repressive histone modifications, the H3K27me3 level at the FLOWERING LOCUS C region, and other polycomb repressive complex 2 targets as well as at heterochromatic retroelements (Hyun et al. 2013). Therefore, the maintenance of repressive epigenetic marks in *ETT*/*ARF3* might be disrupted in the *icu2-1* mutant. No enrichment of H3K27me3 was detected, however, at the *ETT*/*ARF3* promoter regions, the AS1–AS2 binding site, or within the *ETT*/*ARF3* coding sequence (Husbands et al. 2015; Iwasaki et al. 2013; Roudier et al. 2011; Zhang et al. 2007).

AS1–AS2 is involved in maintaining levels of CpG methylation in the *ETT*/*ARF3* coding region, suggesting that AS1–AS2 plays a key role in epigenetic repression of *ETT*/*ARF3* (Iwasaki et al. 2013). Methylation at these CpG sites were abolished in the mutant of *MET1* and the transcript level of *ETT*/*ARF3* in shoot apices was increased (Iwasaki et al. 2013). *MET1* is an *Arabidopsis* homolog of vertebrate DNA methyltransferase1 (DNMT1) (Finnegan et al. 1996), which is responsible for maintaining methylated CpGs during DNA replication (Long et al. 2013). Molecular mechanisms of the DNA replication-coupled maintenance of methylated CpGs in mammalian cells have recently been reported (Ferry et al. 2017; Nishiyama et al. 2013). Mechanisms involving the cooperative action of AS1–AS2 and the replication machinery including ICU2, RFC3 and newly identified factors might be involved in epigenetic repression of the *ETT*/*ARF3* gene that is critical for the formation of flat and symmetric leaves. Recently, we have shown that some modifier genes for the AS2 function are implicated in maintaining CpG methylation in the *ETT*/*ARF3* gene. It should be intriguing to investigate the molecular mechanism of epigenetic regulation of the *ETT*/*ARF3* gene by the cooperative action AS1–AS2 and replication factors such as *ICU2* and *RFC3* to form flat symmetric leaves.

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Table S1. Sequences of primers used for real-time RT-PCR

Primers	Sequences
ETT-F	ATCATTGAGATTCCAGAGGGTCTT
ETT-R	GGCTCCACCATCCGAACA
ARF4-F	CAGGTGTTATGGACCTGGATAGG
ARF4-R	CCAGCAAATTGCGGGAAT
KAN2-F	AAGGAACTAGATGGAAAGTGCTCAA
KAN2-R	GCTTGTTCCCGAGATGCTTG
FIL-F	GCCCACTTCCCCCACATAC
FIL-R	TTGGTTTTCTTCACGGGTTGA
YAB5-F	ACGCCCTAATTTCCAGGCAAC
YAB5-R	GTTGCTCAGTTATGGTACGAG
PHB-F	GCTGTTGACTGGGTTCAGATGA
PHB-R	GCGAAATAGCGACTATGCCAAT
BP-F	TGTTGTTTCCACATATGAGCTCTCT
BP-R	TCATGATCAGATCGGAAGCAAT
KNAT2-F	TTCCGCTCGACGGAAGAC
KNAT2-R	AATCGGACGGCATCATCAAC
STM-F	CTCCTCCCCAAGGAACTAAGAAC
STM-R	TCCTCCTGCAACGATTTCG
ACT2-F1	TCGTTTCTATGATGCACTTGTGTGT
ACT2-R1	ACAAAGGAATAAAGAGGCATCAAT