Development 135, 1325-1334 (2008) doi:10.1242/dev.017913

Ribosomal proteins promote leaf adaxial identity

Yao Yao*, Qihua Ling*, Hua Wang and Hai Huang†

Establishing abaxial-adaxial polarity is central to leaf morphogenesis and function. Groups of genes that encode different components for leaf patterning have been identified in recent years. These include transcriptional factors, small RNAs, 26S proteasome and components required for post-transcriptional gene silencing and chromatin remodeling, showing a complex regulatory network and indicating that the regulation occurs at different levels. In this work, we report the identification and characterization of *asymmetric leaves1/2 enhancer5* (*ae5*) and *ae6* mutants. These two mutants had a phenotype of abnormal leaf patterning, with the abaxial mesophyll features appearing in the adaxial mesophyll domain, and double mutants *ae5 as1/2* and *ae6 as1/2* producing severely abaxialized leaves. *AE5* and *AE6* encode the ribosomal large subunit proteins RPL28A and RPL5A, respectively, and mutations in two other ribosomal protein genes, *RPL5B* and *RPL24B*, resulted in plant phenotypes similar to those of *ae5* and *ae6*. Because these four ribosomal proteins are located in distinct sites in the ribosomal large subunit, we propose that the conserved translational function of the ribosome may be required for regulating key components during leaf patterning. Collectively, our data indicate that specific ribosome subunit-mediated translational control is essential in leaf polarity establishment.

KEY WORDS: *Arabidopsis*, *ASYMMETRIC LEAVES1/2*, Leaf development, Polarity establishment, Ribosomal proteins

INTRODUCTION

During leaf development, leaf primordia first initiate as a group of histologically uniform cells from the peripheral zone of the shoot apical meristem. Further development of the leaf primordia results in their patterning along three-dimensional axes: the adaxial-abaxial, proximodistal and mediolateral axes. A series of surgical experiments carried out by Sussex more than 50 years ago (Sussex, 1954; Sussex, 1955) and elaborated recently by Reinhardt et al. (Reinhardt et al., 2005) provide important information that the establishment of the adaxial-abaxial axis is crucial for subsequent leaf development.

An increasing number of genes that regulate leaf adaxial-abaxial polarity have been identified and characterized extensively. Three members in the class III HD-ZIP family, *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*), specify adaxial fate and are expressed in the adaxial domain of leaves (McConnell and Barton, 1998; McConnell et al., 2001; Emery et al., 2003; Zhong and Ye, 2004). Transcripts of these three genes are the targets of microRNA165 and 166 (miR165/166) (Rhoades et al., 2002; Tang et al., 2003). Two other genes, *ASYMMETRIC LEAVES1* (*AS1*) and *AS2*, have also been considered to promote the adaxial identity of leaves by positively regulating *PHB*, *PHV* and *REV* (Lin et al., 2003; Xu et al., 2003). More recently, *AS1*/*AS2* were found to repress miR165/166 so that *PHB*, *PHV* and *REV* transcripts may be stabilized (Li et al., 2005; Fu et al., 2007; Ueno et al., 2007). Recently, genes functioning in the trans-acting siRNA pathway have been uncovered that facilitate adaxial cell fate of leaves, including *RNA-DEPENDENT RNA POLYMERASE6* (*RDR6*), *SUPPRESSOR OF GENE SILENCING3* (*SGS3*), *ZIPPY* (*ZIP*) and *DICER-LIKE4* (*DCL4*) (Peragine et al., 2004; Li et al., 2005; Garcia et al., 2006; Xu et al., 2006).

National Laboratory of Plant Molecular Genetics, Shanghai Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, People's Republic of China.

*These authors contributed equally to this work
†Author for correspondence (e-mail: hhuang@sippe.ac.cn)

Accepted 30 January 2008

In addition to the adaxial promoting components, several genes are required for leaf abaxial identity, including members of the KANADI (KAN) and YABBY (YAB) families (Sawa et al., 1999; Siegfried et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001). KAN genes antagonize class III HD-ZIP genes (Emery et al., 2003), while the YABs are downstream genes of KAN (Eshed et al., 2004). Furthermore, two auxin response factor genes, *AUXIN RESPONSE FACTOR3* (*ARF3*/*ETT*) and *ARF4*, promote abaxial identity (Pekker et al., 2005). *ARF3* and *ARF4* transcripts are the targets of a small RNA, termed tasiR-ARF (Allen et al., 2005). Finally, loss of function in 26S proteasome subunit genes results in plants with abaxialized leaves, suggesting that post-translational regulation is required for the normal leaf polarity (Huang et al., 2006).

Here, we implicate a new class of genes, the ribosomal large subunit protein encoding genes, in leaf polarity establishment. In animals, functions of the ribosomal proteins have been investigated extensively, and defects in these proteins can cause various kinds of diseases (Bilanges and Stokoe, 2007; Idol et al., 2007; Scheper et al., 2007) and developmental abnormalities (Oliver et al., 2004; Uechi et al., 2006; Marygold et al., 2007). Compared with those in animals, only a few ribosomal proteins in plants have been characterized. *POINTED FIRST LEAVES1* (*PFL1*) and *PFL2* encode the PRS18 and PRS13 ribosomal small subunit proteins, respectively, playing roles in leaf development (Van Lijsebettens et al., 1994; Ito et al., 2000). A semi-dominant mutation of the *Arabidopsis MINUTE-LIKE1* gene, which encodes an additional ribosomal small subunit RPS5, led to early embryonic developmental defects by disrupting cell division (Weijers et al., 2001). Finally, loss of function in the *SHORT VALVE1* (*STV1*) gene, which encodes a ribosomal large subunit protein RPL24B, resulted in the apical-basal patterning defect of gynoecium by influencing *ARF3* translation (Nishimura et al., 2005). All these results indicate that ribosomal proteins are widely involved in different plant developmental processes.

In the present study, we report characterizations of two ribosomal large subunit genes, *RPL28A* and *RPL5A*, both of which play important roles in specifying leaf adaxial identity. Moreover, we found that other two ribosomal protein genes, *STV1* and *RPL5B*, also have this function in leaf pattern formation, indicating that specific

ribosomal functions are required for proper leaf patterning. In addition, we present detailed analyses of genetic interactions between the ribosomal pathway and other genetic pathways during leaf polarity formation.

MATERIALS AND METHODS

Plant materials and growth conditions

The *as1-101*, *as2-101* and *rdr6-3* mutants were generated as described previously (Sun et al., 2000; Sun et al., 2002; Xu et al., 2002; Li et al., 2005). For generation of *as2* enhancers, about 8000 *as2-101* seeds (L*er*) were ethyl methanesulfonate mutagenized (0.15%), and the seeds from 2749 individual M2 lines were screened. Two *as2* enhancer mutants, *ae5-1* and *ae6-1*, were obtained, which were backcrossed to wild-type L*er* three times before detailed phenotypic analyses. Seeds of *ae5-2* (SALK_138179), *ae6-2* (SALK_089798), *rpl5b* (SALK_010121) and *arf4-2* (SALK_070506) were obtained from the ABRC (Ohio State University, Columbus, OH, USA). Seeds of *rev-9, kan1-2/kan1-2 kan2-1*/+ were provided by J. Bowman (University of California, Davis). Seeds of *stv1-1* and *ett-3* were from K. Okada (Nishimura et al., 2005) and P. Zambryski (University of California, Berkeley), respectively. Plant growth was carried out according to our previous conditions (Chen et al., 2000).

RT-PCR

RNA extraction was performed as described previously (Xu et al., 2003) with leaves from 25-day-old seedlings, and reverse transcription was performed with 1 μg total RNA using a kit (Fermentas, Vilnius, Lithuania). PCR was performed with the following gene-specific primers: 5′-ATGGCGACAGTTCCAGGAC-3′ and 5′-TTAAGCTTGTCTGTTTC-TTTG-3′ for *RPL28A*; 5′-TGTGAACACAAAAGCTGAGTG-3′ and 5′-CAACCAAGACAAGAACAAGTAC-3′ for *RPL5A*; and 5′-TGGCATC-A(T/C)ACTTTCTACAA-3′ and 5′-CCACCACT(G/A/T)AGCACA-ATGTT-3′ for *ACTIN*. PCR was performed according to our previous methods (Li et al., 2005).

In situ hybridization and microscopy

In situ hybridization was performed according to a previously described method (Long and Barton, 1998) using 14-day-old seedlings. *FIL* and *REV* probe preparations were according to our previous methods (Li et al., 2005). *AE5* and *AE6* probes were made from cDNA clones containing sequences from exon 4 of *AE5* and exon 7 of *AE6*, respectively. Microscopic analyses were performed according to the previously described methods (Chen et al., 2000; Sun et al., 2002).

For confocal laser-scanning microscopy, the *YFP-RPL28A* and *YFP-RPL5A* fusions were first constructed. The *YFP-RPL28A* and *YFP-RPL5A* fragments were then subcloned into vector pER8 (Zuo et al., 2000), resulting in *pER8-YFP-RPL28A* and *pER8-YFP-RPL5A*, respectively. These two constructs were introduced into wild-type Col-0 plants by *Agrobacterium*-mediated transformation, and the 10-day-old seedlings of the transgenic T1 plants were used for observation after pretreatment with 4 μM estradiol for 3 hours. Specimens were viewed with an LSM510 laser-scanning confocal microscope (Zeiss, Jena, Germany) at a wavelength of 514 nm (YFP).

RESULTS

Identification of new leaf polarity-controlling genes *RPL28A* and *RPL5A*

In a genetic screen for enhancers and suppressors of the *as2-101* mutant, we identified two mutant plants showing similar enhanced *as2* phenotypes. Further genetic analyses of these two mutants resulted in the isolation of two candidate single *as2* enhancer mutants, designated *ae5-1* and *ae6-1* for *asymmetric leaves enhancer*. Of these two mutants, *ae5-1* had pale green leaves while *ae6-1* showed normal plant phenotypes. The identified candidate *ae5* and *ae6* single mutants were then crossed back to *as2-101*, respectively, and the plants with the similar enhanced *as2* phenotypes were observed in both F2 progeny.

To identify the *AE5* and *AE6* genes, we carried out map-based cloning by crossing *ae5-1 as2-101* and *ae6-1 as2-101* to wild-type Col-0 plants. Using about 2000 recombinant chromosomes each, we mapped *AE5* and *AE6* to the lower arm of chromosome 2 and the upper arm of chromosome 3 in a less than 30 kb region, respectively (Fig. 1A,B). We sequenced coding regions of all putative genes in the *ae5* region and identified a gene containing a G-to-A substitution in the second exon, resulting in a premature stop codon (Fig. 1A). This gene (At2g19730) encodes RPL28A, a protein in the large subunit of the ribosome. We then analyzed the *ae6* mapping region and found another ribosomal protein coding gene (At3g25520), *RPL5A* (Fig. 1B). Although sequencing of the *RPL5A*-coding region did not show any change in the nucleotide sequence, the expression level of the *RPL5A* gene was dramatically reduced (see Fig. S1 in the supplementary material). Subsequent analyses revealed that the 3′ region of the gene was disrupted by an *Arabidopsis* transposon *Tag1* (Tsay et al., 1993), which may cause mRNA instability of this gene (see Fig. S1 in the supplementary material).

To further confirm by complementation that we had identified the correct genes, we transformed the *ae5-1 as2-101* mutant with a complementation construct containing 1.3 kb of the *RPL28A* gene plus 1.9 kb and 1.1 kb of its 5′ and 3′ regions (see Fig. S2A in the supplementary material). We also transformed the *ae6-1 as2-101* mutant with a construct containing 1.8 kb of the *RPL5A* gene together with 1.9 kb and 0.8 kb of the 5′ and 3′ regions (supplementary material Fig. S2B). A total of 20 and 25 transgenic lines, respectively, were obtained, of which all plants showed only *as2-101* single mutant phenotypes (see Fig. S2C-F in the supplementary material), indicating that *RPL28A* and *RPL5A* indeed correspond to the enhanced *as2* phenotypes in the *ae5-1 as2-101* and *ae6-1 as2-101* mutant plants.

RT-PCR revealed that *AE5* and *AE6* were expressed in all plant tissues examined (Fig. 1C). In situ hybridization using the gene-specific sequence as probes showed that *AE5* and *AE6* transcripts were detected throughout the embryo (Fig. 1D,E) and leaf primordia (Fig. 1F,G) at earlier developmental stages. The same expression pattern was also detected in the reproductive organs, with the hybridization signals being throughout the inflorescence meristem, floral primordia and four types of young floral organs (Fig. 1H). The in situ hybridization experiments were performed with two types of negative controls: (1) sense probes (Fig. 1I); and (2) an insertional *ae6-2* allele (Fig. 1J; for identification of *ae6-2*, see Fig. S3 in the supplementary material). These results indicate that *RPL28A* and *RPL5A* are new regulators of leaf polarity despite their expression throughout leaf primordia.

Mutations of *ae5* and *ae6* affect leaf adaxial-abaxial polarity

To understand the functions of *AE5* and *AE6* in leaf development, we analyzed phenotypes of *ae5/6 as1* and *ae5/6 as2* mutants, as well as *ae5* and *ae6* single mutants. Compared with wild-type (Fig. 2A) and *as2-101* (Fig. 2B) plants, both *ae5-1 as2-101* (Fig. 2D) and *ae6-1 as2-101* (Fig. 2E) displayed an increased number of lotus- and needle-like leaves (Table 1). Of these two double mutants, the *ae5-1 as2-101* phenotypes appeared even more severe with almost all true leaves being needle-like in a proportion of the seedlings (Fig. 2D). Although most first two leaves in the *ae6-1 as2-101* mutant were expanded, the adaxial surface of the leaves was rough (Fig. 2E). For the single mutants, *ae6-1* had a phenotype similar to that of wild-type plants (Fig. 2G), whereas the early appearing leaves of *ae5-1* were slightly longer and all leaves were pale green (Fig. 2F).

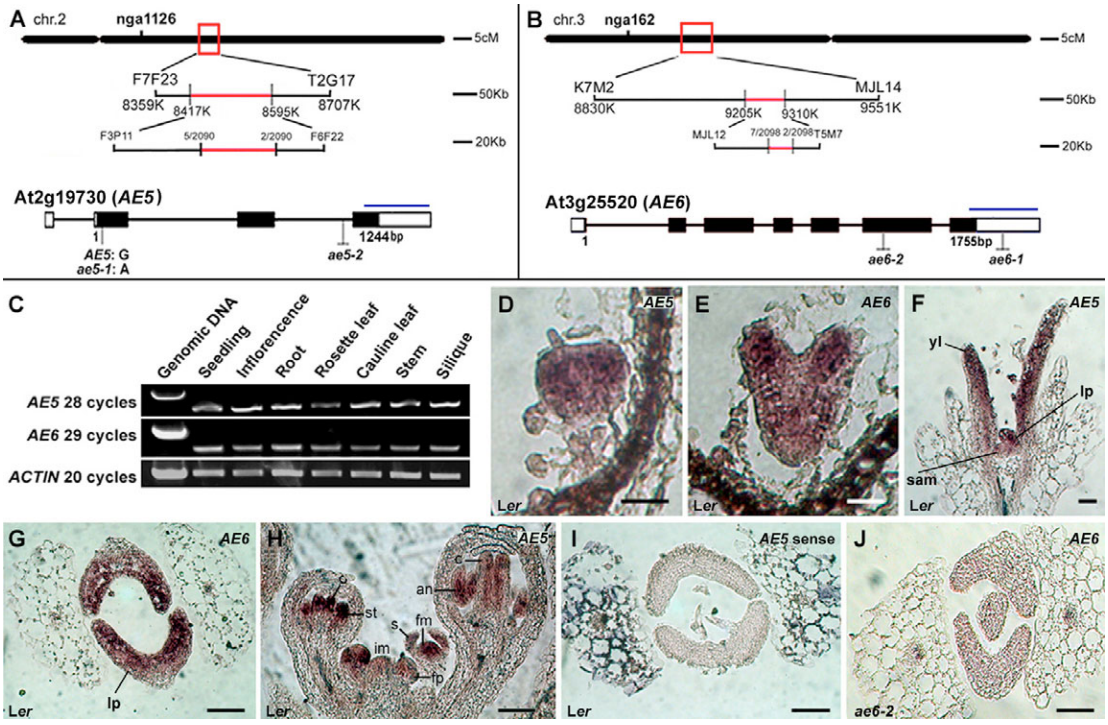

Fig. 1. Molecular identification of *AE5* and *AE6*. (**A**,**B**) Fine structure mapping to localize *AE5* and *AE6* genes on chromosome 2 (A, upper panel) and 3 (B, upper panel), and *AE5* (At2g19730) and *AE6* (At3g25520) encode ribosomal proteins RPL28A (A, lower panel) and RPL5A (B, lower panel), respectively. Black and white boxes indicate the protein-coding region and untranslational region (UTR), respectively. (**C**) RT-PCR shows that *AE5* and *AE6* were expressed in all plant tissues examined. (**D-J**) In situ hybridization analyses to determine *AE5* (D,F,H) and *AE6* (E,G) expression patterns. (D) A late-globular-stage embryo. (E) A heart-stage embryo. (F) A longitudinal section of an 11-day-old seedling. (G) A transverse section of an 11-day-old seedling. (H) A longitudinal section of an inflorescence meristem. (I,J) No hybridization signals were detected either with the *AE5* sense probe (I) or with the *AE6* antisense probe for tissues from the insertional allele, *ae6-2* (J). Blue lines in A,B indicate probe regions used for in situ hybridization. sam, shoot apical meristem; lp, leaf primordium; yl, young leaf; im, inflorescence meristem; fm, floral meristem; fp, flower primordium; s, sepal; st, stamen; c, carpel; an, anther. Scale bars: 25 μm in D,E; 100 μm in F-J.

To determine whether the *ae5-1* and *ae6-1* mutations can also enhance *as1* phenotypes (Fig. 2C), we constructed *ae5-1 as1-101* and *ae6-1 as1-101* double mutants. These two double mutants (Fig. 2H,I) had leaf phenotypes very similar to those of *ae5-1 as2-101* (Fig. 2D) and *ae6-1 as2-101* (Fig. 2E), respectively. In addition, we identified an additional allele for both *ae5* and *ae6*, here referred to as *ae5-2* and *ae6-2*, from the SALK insertional collection (see Fig. S3 in the supplementary material). Both *ae5-2* (Fig. 2J) and *ae6-2* (Fig. 2L) are in the Col-0 background and possessed pale green leaves, similar to those in *ae5-1*. Double mutant plants combining *as2-1* (Col-0, data not shown) with *ae5-2* or *ae6-2* both strongly enhanced the *as2-1* leaf phenotypes (Fig. 2K,M), producing many needle-like rosette leaves. These results indicate that the AS1/AS2 and the ribosomal pathways act in parallel in promoting leaf adaxial cell fates.

Multiple developmental defects in *ae5* and *ae6* single mutants

To characterize in more detail the developmental abnormalities of the *ae5* and *ae6* mutants, we analyzed phenotypes of two severe alleles, *ae5-1* and *ae6-2*. Compared with the wild-type plants (Fig. 3A), although primordia of the first two rosette leaves in the mutants emerged normally (data not shown), subsequent leaf growth was delayed in *ae5-1* (Fig. 3B) and *ae6-2* (data not shown). In the first pair of fully expanded rosette leaves of wild-type plants, the lateral veins were evident only in the leaf blade (Fig. 3C,D). By contrast, vein branching in the *ae5-1* (Fig. 3E, inset) and *ae6-2* (Fig. 3F, inset) occurred more proximally in the petiole. In addition, the number of small veins that appear at later leaf developmental stages was reduced (Fig. 3E,F).

To determine the anatomical basis of the pale green leaves of *ae5-1* and *ae6-2* mutants, we analyzed their lamina structure by transverse sectioning. In wild-type L*er* (Fig. 3G) and Col-0 (Fig. 3H) plants, four distinct cell types in the lamina of expanded leaves are recognizable along the adaxial-abaxial axis: adaxial epidermis, palisade mesophyll, spongy mesophyll and abaxial epidermis. Of the two types of mesophyll cells, the adaxially located palisade cells are usually tightly arranged, whereas the abaxially positioned spongy cells are loosely arranged with intercellular spaces (Fig. 3G,H, arrows). However, in the *ae5-1* and *ae6-2* leaves, numerous intercellular spaces occurred in the adaxial palisade region (Fig. 3I,J, arrowheads), reflecting a disrupted leaf adaxial-abaxial axis. These results also suggest that the less tightly arranged palisade cells at least partially contribute to the pale green color of the *ae5-1* and *ae6-2* leaves.

Compared with the inflorescences in wild-type plants (Fig. 3K), those from some *ae5-1* (Fig. 3L,M) and *ae6-2* (data not shown) plants were abnormal. The inflorescence of some *ae5-1* plants terminated early, by producing several secondary inflorescences (Fig. 3L). In wild-type plants, a secondary inflorescence is usually associated with a cauline leaf at its proximal end (Fig. 3K), whereas in *ae5-1* plants a proportion of

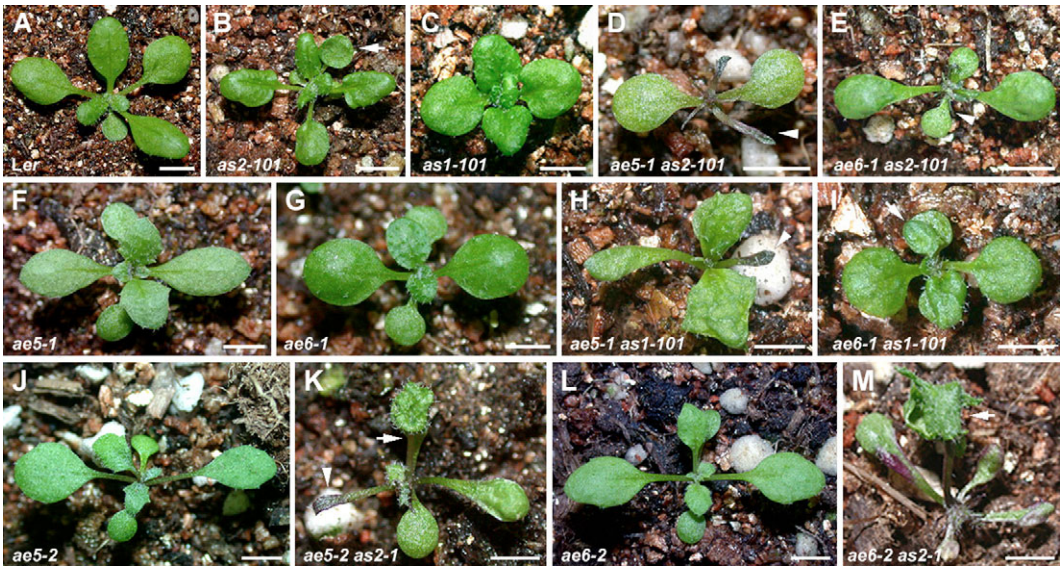

Fig. 2. *ae5* and *ae6* enhance *as2* and *as1* leaf polarity defects. (**A-I**) Phenotypes of wild-type and mutant seedlings in the L*er* background. (A) Wild-type L*er*. (B) *as2-101*, (C) *as1-101*. (D) *ae5-1 as2-101*. (E) *ae6-1 as2-101*. (F) *ae5-1*. (G) *ae6-1*. (H) *ae5-1 as1-101*. (I) *ae6-1 as1-101*. (**J-M**) Phenotypes of mutant seedlings in the Col-0 background. (J) *ae5-2*. (K) *ae5-2 as2-1*. (L) *ae6-2*. (M) *ae6-2 as2-1*. Arrows indicate the lotus-like leaves, and arrowheads indicate needle-like leaves. Scale bars: 0.5 cm.

secondary inflorescences lacked a subtending cauline leaf (Fig. 3M, arrowhead). Occasionally, some cauline leaves in *ae5-1* (Fig. 3N) and *ae6-2* (data not shown) formed ectopic outgrowths on their abaxial distal parts. All these results indicate that *RPL28A* and *RPL5A* are involved in multiple plant developmental processes.

ae5 as2 and *ae6 as2* double mutants produce severely abaxialized leaves

To further understand the roles of *RPL28A* and *RPL5A* in leaf patterning, we analyzed leaf phenotypes of *ae5 as2* and *ae6 as2* double mutants by SEM. The double mutant plants produced three types of leaves from weak to strong phenotypes: (1) expanded

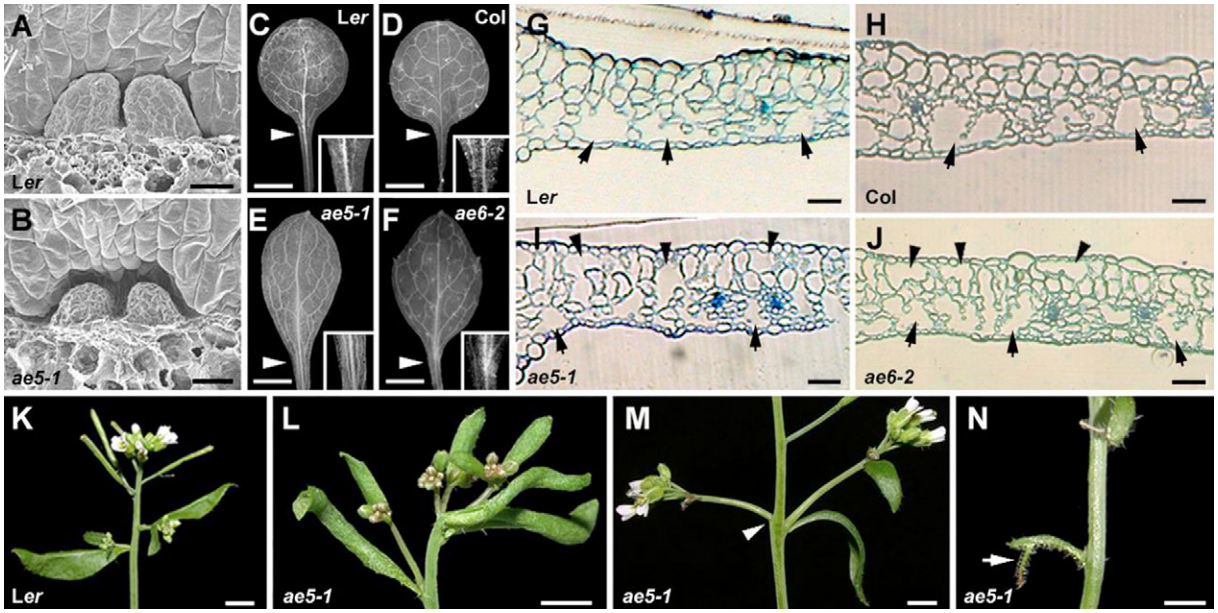

Fig. 3. Phenotypes of the *ae5-1* and *ae6-2* single mutants. (**A**,**B**) Primordia of the first two leaves in 3-day-old wild-type (A) and *ae5-1* (B) seedlings. (**C-F**) Venations of the first-pair rosette leaves in wild-type L*er* (C), wild-type Col-0 (D), *ae5-1* (E) and *ae6-2* (F). Insets in C-F show higher magnification of the vein pattern. (**G-J**) Transverse sections of the leaf blade in L*er* (G), Col-0 (H), *ae5-1* (I) and *ae6-2* (J). Arrows indicate the normal abaxial intercellular spaces, while arrowheads show the ectopically positioned intercellular spaces. (**K**) An inflorescence of a wild-type L*er* plant. (**L-N**) Inflorescences of *ae5-1* showed various abnormalities, including disorganized inflorescences (L), abnormal phyllotaxy (M) and an ectopic leaf outgrowth that formed on distal part of the abaxial side of some cauline leaves (N, arrow). The arrowhead in M indicates two associated lateral branches; a cauline leaf was absent at the proximal end of one branch. Scale bars: 50 μm in A,B; 0.3 cm in C-F; 30 μm in G-J; 0.5 cm in K-N.

Table 1. Frequencies of lotus- and needle-like leaves in the mutant plants*

	First two leaves		Third to sixth leaves	
	Total leaves†	L/N leaves (%)‡	Total leaves†	L/N leaves (%)‡
as2-101	235	19.5	367	0
ae5-1 as2-101	186	46.8	257	86.8
ae6-1 as2-101	202	23.8	316	81.0

*Plants were grown at 22°C.
†Total leaves that were analyzed.
‡Frequency is defined by the ratio of the number of lotus (L)- and needle (N)-like leaves to the total number of corresponding leaves analyzed.

leaves, which were rough on the adaxial lamina surface (Fig. 4A, arrow); (2) lotus-like leaves, in which the petiole was centrally attached underneath the blade (Fig. 4B); and (3) needle-like leaves (Fig. 4C). In wild-type plants, epidermal cells of the adaxial lamina surface are relatively large and of uniform size (Fig. 4D), whereas those of the abaxial side are smaller and mixed with some long and narrow cells (Fig. 4E, arrowheads). The epidermal cell patterns of the *ae5-1* (see Fig. S4 in the supplementary material) and *ae6-1* (data not show) single mutant leaves were normal. However, the adaxial surface of the expanded leaves in *ae5-1 as2-101* (Fig. 4A) and *ae6-1 as2-101* (data not shown) was a mosaic containing some normal adaxial (Fig. 4F) and abnormal abaxial (Fig. 4G) epidermal cell patches. Most epidermal cells on the needle-like leaves were rectangular (Fig. 4H), except those on the apical part of the leaves, which showed partial differentiation with the abaxially featured long cells (Fig. 4I, arrowhead).

In the blade-petiole junction region of wild-type leaves, vascular bundles showed a pattern where xylem develops on the adaxial pole and phloem is located on the abaxial pole (Fig. 4J). In the expanded leaves of the weak allele *ae6-1 as2-101*, adaxial extension of the phloem was apparent (Fig. 4K). By contrast, in the lotus-like leaf of the more severe *ae5-1 as2-101* allele, the vascular bundle structure comprised a concentric ring of phloem surrounding the least-developed xylem cells in the center (Fig. 4L). This is a typical phenotype of the abaxialized leaf, and this phenotype supports the idea that *AS1/2* and *RPL28A/5A* are required for promoting leaf adaxial cell fate.

To obtain molecular evidence for the RPL28A and RPL5A function in specifying leaf polarity, we examined by in situ hybridization the expression pattern of two marker genes, *FIL* and *REV*. In wild-type plants, *FIL* is usually expressed on the abaxial side of leaves (Siegfried et al., 1999) (Fig. 5A), and this pattern was

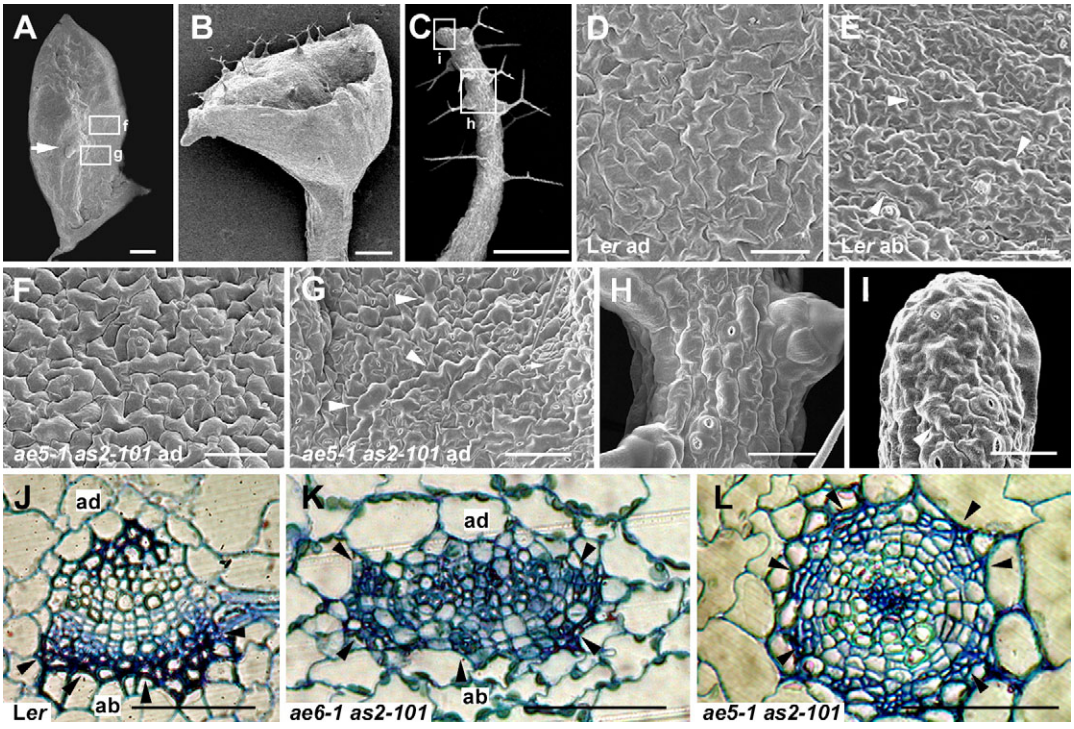

Fig. 4. Aberrant adaxial-abaxial polarity in leaves of *ae5-1 as2-101* and *ae6-1 as2-101*. (**A-C**) Three types of abnormal rosette leaves in *ae5-1 as2-101*. (A) An adaxial view of an expanded leaf showing a rough surface and some bumps (arrow). (B) A lotus-like leaf. (C) A needle-like leaf. (**D-I**) Analyses of the leaf epidermal patterns. (D,E) Epidermal cells on adaxial (D) and abaxial (E) surfaces of a wild-type leaf. (F,G) High magnification of epidermal cells in A. The same adaxial leaf side of *ae5-1 as2-101* contained both adaxially (F) and abaxially (G) featured epidermal cells, which correspond to the boxed regions f and g in A, respectively. (H,I) High magnification of epidermal cells in C, corresponding to the boxed regions h and i, respectively. Arrowheads in E,G,I indicate long and narrow abaxially featured epidermal cells. (**J-L**) Transverse sections through the blade-petiole junction region. (J) A section from a wild-type L*er* leaf. (K) A section from an expanded *ae6-1 as2-101* leaf. (L) A section from an *ae5-1 as2-101* lotus-like leaf. Arrowheads in J-L indicate phloem. ad, leaf adaxial side; ab, leaf abaxial side. Scale bars: 1 mm in A-C; 50 μm in D-L.

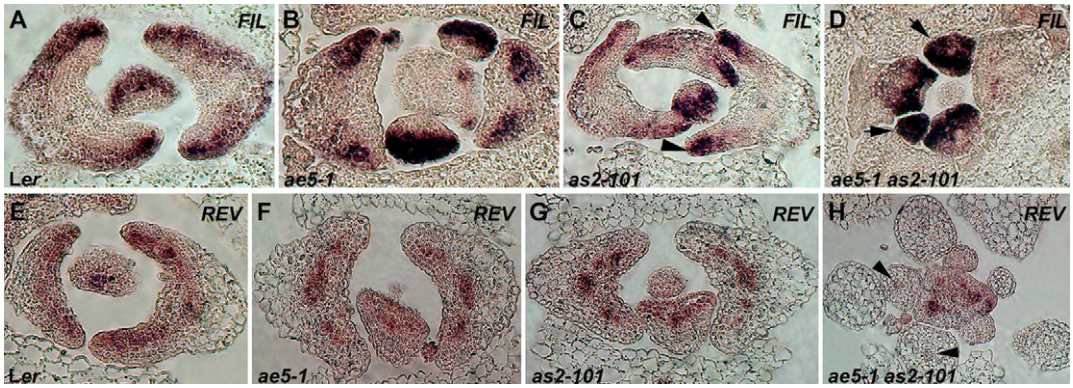

Fig. 5. In situ localization of *FIL* and *REV* in *ae5-1* and *ae5-1 as2-101* mutants. (**A-D**) In situ hybridization of *FIL* in L*er* (A), *ae5-1* (B), *as2-101* (C) and *ae5-1 as2-101* (D). (**E-H**) In situ hybridization of *REV* in L*er* (E), *ae5-1* (F), *as2-101* (G) and *ae5-1 as2-101* (H). The *FIL* expression was adaxially extended in some primordia of *as2-101* (C, arrowheads) and was apparent throughout some primordia of *ae5-1 as2-101* (D, arrows). Arrowheads in H indicate vascular tissues.

also observed in *ae5-1* leaves (Fig. 5B). *FIL* expression appeared to be extended towards the adaxial side in some young leaves in *as2-101* (Fig. 5C, arrowheads), and was further expanded throughout some needle-like leaf primordia of *ae5-1 as2-101* (Fig. 5D, arrows; see Fig. S5 in the supplementary material). *REV* is known to be expressed in the adaxial domain of leaf primordia as well as in the vascular tissue of developing leaves (Eshed et al., 2001) (Fig. 5E). Although the *REV* expression pattern did not show obvious changes between the wild type (Fig. 5E), *ae5-1* (Fig. 5F) and *as2-101* (Fig. 5G), the normal *REV* expression pattern was not apparent in leaf

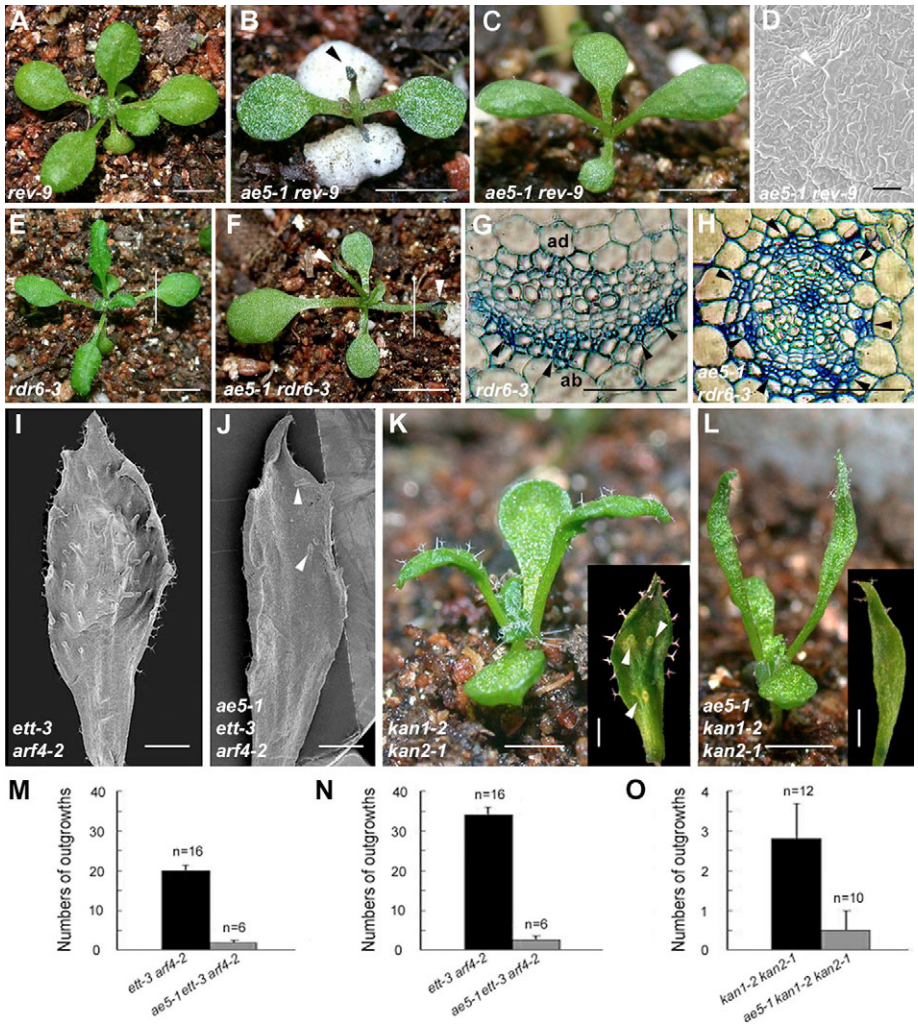

Fig. 6. Phenotypic analyses of double and triple mutants. (**A**) A *rev-9* seedling. (**B**) A severe *ae5-1 rev-9* seedling with only expanded cotyledons. An arrowhead indicates a needle-like leaf. (**C**) A less severe *ae5-1 rev-9* seedling showing rosette leaves with rough leaf surface. (**D**) SEM of adaxial leaf surface of *ae5-1 rev-9*, showing that the abaxially featured epidermal cells appeared on the adaxial leaf side (arrowhead). (**E**) An *rdr6-3* seedling. (**F**) An *ae5-1 rdr6-3* seedling with lotus leaves (arrowheads). (**G**,**H**) Transverse sections of *rdr6-3* (G) and *ae5-1 rdr6-3* (H) leaves. The positions of sectioning are indicated by white lines in E and F. Arrowheads in G,H indicate the phloem. (**I**) SEM of a leaf from *ett-3 arf4-2* double mutant, showing a number of outgrowths on the abaxial leaf side. (**J**) SEM of an *ae5-1 ett-3 arf4-2* leaf, exhibiting a dramatically reduced number of the outgrowths. (**K**) A *kan1-2 kan2-1* seedling. (**L**) An *ae5-1 kan1-2 kan2-1* seedling. Insets in K,L show the abaxial surface of the first-pair leaf; arrowheads in J,K indicate the outgrowths. (**M**,**N**) Quantitative analyses of outgrowths on the third-pair (M) and fourth-pair (N) rosette leaves of *ett-3 arf4-2* and *ae5-1 ett-3 arf4-2*. (**O**) Quantitative analysis of outgrowths on the first-pair rosette leaves of *kan1-2 kan2-1* and *ae5-1 kan1-2 kan2-1*. Columns and error bars in M-O represent the mean and s.d. Scale bars: 0.5 cm in A-C,E,F; 50 μm in D,G,H; 3 mm in I,J; 2 mm in K,L; 1 mm in K,L insets.

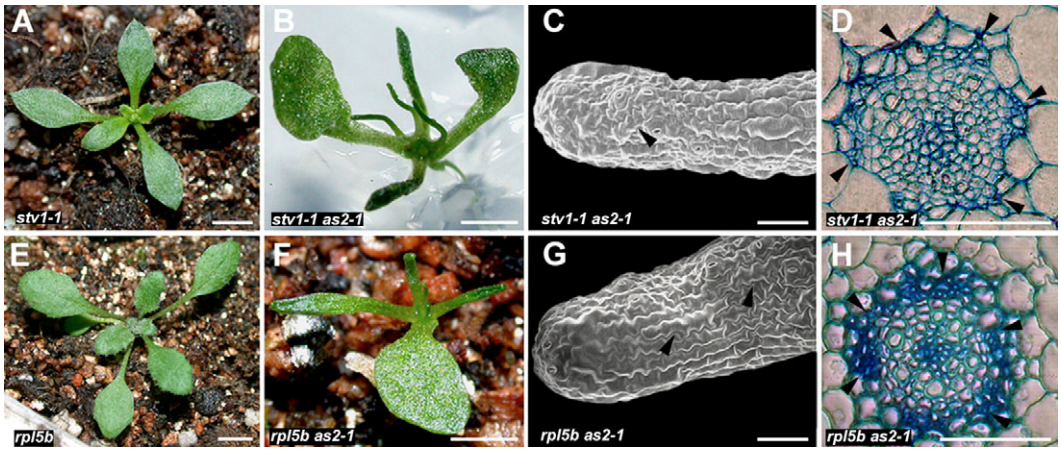

Fig. 7. Mutations in *RPL24B* and *RPL5B* enhance *as2-1* polarity defects. (**A**) An *stv1-1* seedling. (**B**) An *stv1-1 as2-1* seedling. (**C**) SEM to examine epidermal cells on the top part of an *stv1-1 as2-1* needle-like leaf. (**D**) Transverse section of an *stv1-1 as2-1* needle-like leaf petiole. (**E**) An *rpl5b* seedling. (**F**) An *rpl5b as2-1* seedling. (**G**) The top part of an *rpl5b as2-1* needle-like leaf. (**H**) Transverse section of an *rpl5b as2-1* needle-like leaf petiole. Arrowheads in C,G show the abaxially featured epidermal cells. Arrowheads in D,H indicate phloem. Scale bars: 0.5 cm in A,B,E,F; 50 μm in C,D,G,H.

primordia of *ae5-1 as2-101* (Fig. 5H). In addition, the *REV* signal was markedly reduced in the primordia of the double mutant, whereas in vascular bundles the *REV* expression level appeared close to that in the wild-type primordia (Fig. 5H; see Fig. S5 in the supplementary material). These results indicate that ribosomal proteins are required genetically for regulation of the key leaf polarity-controlling genes during leaf patterning.

Genetic interactions between *RPL28A/5A* and other polarity genes

To determine possible genetic interactions between *RPL28A/5A* and other leaf polarity-controlling genes, we constructed double and triple mutants and analyzed their phenotypes. The recessive *rev-9* has normally patterned leaves (Fig. 6A), whereas *ae5-1 rev-9* showed severe leaf phenotypes. The shoot apical meristem of about 20% seedlings was terminated after a few lotus- or needle-like leaves were produced (Fig. 6B). Although other seedlings continued to grow, their rosette leaves became rough (Fig. 6C). SEM analysis showed that there were ectopic abaxial epidermal cells on the adaxial surfaces of the rosette leaves (Fig. 6D, arrowhead), suggesting that these leaves are abaxialized. Similarly, leaves of the *rdr6* single mutant exhibit minor polarity defects (Peragine et al., 2004) (Fig. 6E), but *ae5-1 rdr6-3* demonstrated more severe defects in leaf polarity, with many lotus- and needle-like leaves (Fig. 6F, arrowheads). The vascular pattern through the blade-petiole junction region in *rdr6-3* (Fig. 6G) was similar to that of wild-type plant (Fig. 4J), whereas that in *ae5-1 rdr6-3* (Fig. 6H) was apparently abaxilized, with a phloem-surrounding-xylem structure.

Double mutants *arf3 arf4* and *kan1 kan2* have some similar leaf phenotypes (Pekker et al., 2005), with narrow and dark green rosette leaves and ectopic outgrowths on the abaxial leaf side (Fig. 6I,K). Interestingly, on abaxial surfaces of the *ae5-1 ett-3 arf4-2* (Fig. 6J,M,N) and *ae6-2 ett-3 arf4-2* (data not show) triple mutant leaves, the number of the outgrowths was dramatically decreased. The *ae5-1 kan1-2 kan2-1* triple mutant only had two very narrow leaves, with other leaves being arrested at their earlier developmental stages (Fig. 6L). Similarly, the number of the outgrowths on abaxial surfaces of the two *ae5-1 kan1-2 kan2-1* leaves was also reduced (Fig. 6L, inset), when compared with those of *kan1-2 kan2-1* (Fig. 6K, inset, Fig. 6O). The reduction of the outgrowth numbers in the triple mutants was also accompanied by the impaired severity of outgrowth shapes (see Fig. S4 in the supplementary material). These results indicate that *RPL28A* and *RPL5A* may genetically interact with the *KAN1/2* and *ARF3/4* pathways to regulate leaf polarity.

Leaf polarity defects in *stv1 as2* and *rpl5b as2* double mutants

To determine whether other ribosomal subunit genes also participate in leaf patterning, we constructed *stv1 as2* and *rpl5b as2* double mutants. *STV1* encodes a ribosomal large subunit protein RPL24B (Nishimura et al., 2005). Although *stv1-1* (Fig. 7A) and *rpl5b* (Fig. 7E; for identification of *rpl5b*, see supplementary material Fig. S3) both produced pale green leaves, the epidermal cell patterns appeared normal (see Fig. S4 in the supplementary material). By contrast, *stv1-1 as2-1* and *rpl5b as2-1* double mutant plants demonstrated very severe phenotypes with most rosette leaves being needle-like (Fig. 7B,F). These needle-like leaves were covered with rectangular-shaped cells or abaxial-type cells (Fig. 7C,G, arrowheads). Similar to those in the *ae5-1 as2-101* double mutant (Fig. 4L), the vascular pattern of these leaves indicated that they were abaxialized (Fig, 7D,H).

As *RPL5B* is a duplicated copy of *RPL5A* (*AE6*) in the *Arabidopsis* genome, we were interested in determining whether the *ae6 rpl5b* double mutant would more severely affect leaf polarity. Interestingly, the double heterozygote (*ae6-2/+ rpl5b/+*) exhibited a phenotype similar to that of *ae6-2* or *rpl5b* single mutant, with pale green leaves (see Fig. S6 in the supplementary material). However, plants with genotypes *ae6-2/ae6-2 rpl5b/rpl5b*, *ae6-2/+ rpl5b/rpl5b*, or *ae6-2/ae6-2 rpl5b/+* were not found in the F2 progeny of the *ae6-2* and *rpl5b* cross (see Fig. S6 in the supplementary material). Taken together, the enhanced *as2* phenotypes by other ribosomal large subunit gene mutations indicate that the entire ribosomal activity may be required for normal leaf polarity establishment.

Subcellular localization of RPL28A and RPL5A

We next examined the subcellular localization of RPL28A and RPL5A by generating *YELLOW FLUORESCENT PROTEIN* (*YFP*)-*RPL28A* and -*RPL5A* fusions under the control of an

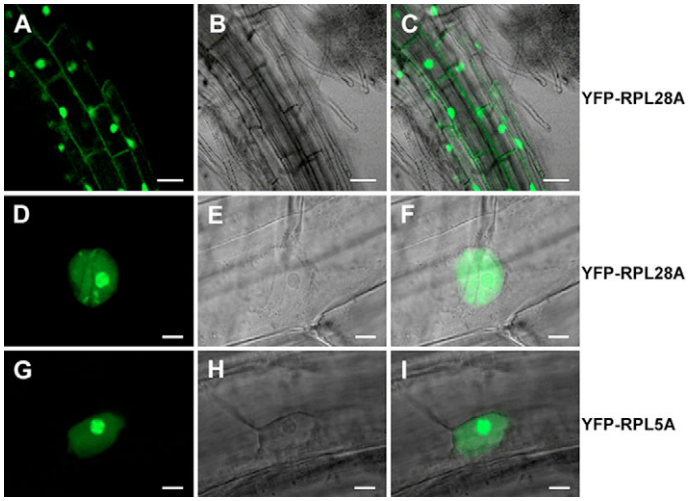

Fig. 8. Subcellular localization of YFP-RPL28A and YFP-RPL5A in the *Arabidopsis* root. (**A-C**) YFP-RPL28A was detected in both nuclei and cytoplasm. (**D-I**) Signals of YFP-RPL28A (D-F) and YFP-RPL5A (G-I) were found in both nucleolus and nucleoplasm. (A,D,G) YFP fluorescence; (B,E,H) bright-field differential interference contrast images; (C,F,I) merged views. Scale bars: 20 μm in A-C; 5 μm in D-I.

estradiol-inducible promoter. These two constructs were introduced into wild-type L*er*, *ae5-1 as2-101* and *ae6-1 as2-101* mutant plants, respectively, and the double mutant phenotypes could be rescued (data not shown), indicating that YFP-RPL28A and YFP-RPL5A are biologically active. In the root tissue of wild-type L*er* plants, YFP-RPL28A (Fig. 8A-C) and YFP-RPL5A (data not shown) were both located in the nuclei and cytoplasm. In addition to the strong YFP signals in the nucleolus, YFP-RPL28A (Fig. 8D-F) and YFP-RPL5A (Fig. 8G-I) were also present in the nucleoplasm. These results indicate that the ribosomal large subunit proteins in plants possess a similar subcellular localization pattern to those in animals (Claussen et al., 1999).

DISCUSSION

Ribosomal proteins are new regulators in specifying leaf adaxial identity

In this paper, we showed that genes encoding ribosomal proteins play important roles in specifying the leaf adaxial identity. Plants carrying a single mutation in the previously characterized leaf adaxial-promoting genes usually results in mild abnormalities in the leaf adaxial-abaxial polarity. For example, plants with loss of function in *REV*, *PHB*, *PHV*, *RDR6*, *SGS3* and *ZIP* either had normal leaves or only showed very minor leaf polarity defects, while such defects in *as1*/*as2* single mutants with the wild-type *ERECTA* were also subtle. Although single mutations in some 26S proteasome subunit genes can lead to the production of needle-like leaves, which is considered to be a strong defect in leaf adaxial-abaxial polarity, the frequency of such leaves is very low (Huang et al., 2006). Unlike other leaf adaxial-promoting factors, the severe *ae5* and *ae6* alleles exhibited a defective phenotype with almost all leaves being pale green in color, and this type of leaves was also observed in *stv1-1* and *rpl5b* single mutants. This phenotype was caused, at least in part, by the ectopic intercellular spaces, which should be normally located in the abaxial spongy mesophyll layer of the wild-type leaves but are present in the adaxial palisade layer of the mutant ones. In addition, double mutants combining *as1*/*as2* with ribosomal protein gene mutations can cause severe defects in the adaxial-abaxial leaf polarity. Our data strongly suggest that ribosomal proteins are a new type of regulator in leaf polarity establishment.

The translational function of the ribosome is required for leaf polarity establishment

The ribosome is composed of two subunits, a large subunit and a small subunit, both of which are formed by ribosomal proteins and rRNAs. The *Arabidopsis* genome contains 227 genes encoding 80 ribosomal proteins, 48 and 32 of which are on the large and small subunits, respectively (Carroll et al., 2007). By interacting with different rRNAs at specific regions, they form different active sites required for the ribosomal function (Ramakrishnan, 2002). In animals, ribosomal proteins are involved in either specific or nonspecific developmental processes (Bilanges and Stokoe, 2007; Ishijima et al., 1998; Idol et al., 2007; Marygold et al., 2007; Oliver et al., 2004; Uechi et al., 2006). For example, knockdown of some ribosomal protein genes in zebrafish resulted in a spectrum of developmental defects with varying degrees of abnormality in the brain, body trunk, eyes and ears (Uechi et al., 2006). In *Drosophila*, 88 genes that encode cytoplasmic ribosomal proteins were characterized, 64 of them were found to correspond to the similar dominant 'Minute' phenotypes (Marygold et al., 2007). Some ribosomal proteins or protein complexes with specific functions were also documented: RPL22 and RPL7 function in transcription repression (Ni et al., 2006); RPL5, RPL11 and RPL23 act in inhibition of protein ubiquitination and degradation (Arva et al., 2005; Dai and Lu, 2004; Dai et al., 2004); and RPL11 is involved in reduction of histone H4 acetylation (Dai et al., 2007).

The functions of four *Arabidopsis* ribosomal proteins have been characterized and the corresponding mutants *pfl1*/*rps18* (Van Lijsebettens et al., 1994), *pfl2*/*rps13* (Ito et al., 2000), *aml1*/*rps5* (Weijers et al., 2001) and *stv1*/*rpl24* (Nishimura et al., 2005) all exhibited pleiotropic developmental defects. However, it was also observed that a loss of functions in different ribosomal protein genes affecting specific developmental processes. For example, *STV1* (*RPL24*) regulates apical-basal patterning of the gynoecium (Nishimura et al., 2005). We propose that regulation of the leaf adaxial-abaxial polarity requires a conserved translational function of the *Arabidopsis* ribosome. First, although RPL28A, RPL5A, RPL5B and RPL24B are all large subunit proteins, according to data from yeast (Spahn et al., 2001) and bacteria (Korostelev et al., 2006; Selmer et al., 2006), these four proteins are located in three distinct sites on the large subunit, whereas plants with mutations in each of these genes suffer similar adaxial-abaxial polarity defects. Second, *pfl2*/*rps13* and *pfl1*/*rps18* also display either pale green leaves (Van Lijsebettens et al., 1994) or the defective mesophyll pattern (Ito et al., 2000), similar to that in the large subunit mutants *ae5*, *ae6*, *stv1* and *rbl5b*. Finally, the double heterozygous plant (*ae6*/+ *rpl5b*/+) exhibited the pale-green leaf phenotype (see Fig. S6 in the supplementary material). These data suggest that a partial loss of the ribosome function may cause some similar leaf polarity defects. As the ribosome is known to be the conserved machinery of translation in all organisms, the regulation of leaf polarity at the translational level must be very important.

Roles of the ribosome in the regulatory network in leaf patterning

The molecular mechanism of how ribosomal proteins regulate leaf polarity-controlling genes is not yet clear. One possibility is that the ribosome may genetically promote the *HD-ZIP III* mediated

pathway in the adaxial domain of leaves. We have two lines of evidence to support this idea: (1) *ae5-1* can enhance a weak *rev* allele, *rev-9*; and (2) accumulation of *REV* transcripts were reduced in *ae5 as2* and *ae6 as2* double mutants. In addition, *AS1/AS2* are known to regulate *PHB*, *PHV* and *REV* positively in leaves (Lin et al., 2003; Fu et al., 2007; Ueno et al., 2007), and *as1/as2* phenotypes could be enhanced in the ribosomal protein gene mutation backgrounds.

Another possibility is that the ribosome may genetically repress *ARF3/4*, *KAN* or their downstream genes. This hypothesis is supported by the observation that *ae5 ett arf4* and *ae6 ett arf4* triple mutants dramatically suppress an *ett arf4* phenotype in which outgrowths are formed on the abaxial leaf side. In addition, *ae5 kan1 kan2* also exhibited the reduction of the outgrowth numbers. It was proposed that juxtaposition of adaxial and abaxial domain is required for lamina outgrowth (Waites and Hudson, 1995), and adaxial and abaxial characteristics on the same abaxial side of the *kan1 kan2* leaves might cause the formation of ectopic outgrowths (Eshed et al., 2004). Based on this proposal, it is possible that the balanced juxtaposition of the adaxial and abaxial cell patches on the same leaf side may be crucial for the outgrowth formation. The addition of *ae5* or *ae6* mutation in the *ett arf4* and *kan1 kan2* backgrounds may alter the original juxtaposition balance by promoting abaxial leaf characteristics, and therefore repressed the outgrowth phenotype.

We thank J. L. Bowman, K. Okada, P. Zambryski and the ABRC (Ohio State University, Columbus, OH, USA) for seeds of mutants *rev-9, kan1-2/kan1-2 kan2-1/+*, *stv1-1*, *ett-3*, *ae5-2* (SALK_138179), *ae6-2* (SALK_089798), *rpl5b* (SALK_010121) and *arf4-2* (SALK_070506). We thank N. Chua for plant expression vector pER8, X. Gao for SEM, and X. Gao for plant sample preparation and sectioning. This research was supported by grants from the Chinese National Scientific Foundation (30630041 and 30721061) and Chinese Academy of Sciences (KSCX2-YW-N-016) to H.H.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/7/1325/DC1

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