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APUM23, a PUF family protein, functions in leaf development and organ polarity in Arabidopsis

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Abstract

The normal biological function of leaves, such as intercepting light and exchanging gasses, relies on proper differentiation of adaxial and abaxial polarity. KANADI (KAN) genes, members of the GAR family, are key regulators of abaxial identity in leaf morphogenesis. This study identified a mutant allele (apum23-3) of APUM23, which encodes a Pumilio/PUF domain protein and acts as an enhancer of the kan mutant. Arabidopsis APUM23 has been shown to function in pre-rRNA processing and play pleiotropic roles in plant development. The apum23-3 mutant also synergistically interacts with other leaf polarity mutants, affects proliferation of division-competent cells, and alters the expression of important leaf polarity genes. These phenotypes show that APUM23 has critical functions in plant development, particularly in polarity formation. The PUF gene family is conserved across kingdoms yet it has not been well characterized in plants. These results illuminating the functions of APUM23 suggest a novel role for PUF genes in Arabidopsis leaf development.

Key words: APUM23, Arabidopsis, KANADI, leaf development, polarity, PUF.

Introduction

The leaves of many plant species exhibit differences between their adaxial (dorsal) and abaxial (ventral) surfaces (Telfer and Poethig, 1994). Proper specification of adaxial and abaxial identity is required for the formation and function of leaves. In Arabidopsis thaliana, adaxial identity is specified by the class III homeodomain-leucine zipper (HD-ZIPIII) genes (McConnell et al., 2001; Emery et al., 2003; Prigge et al., 2005), Myb and LOB domain transcription factors ASYMMETRIC LEAVES1 (AS1) and AS2 (Lin et al., 2003; Xu et al., 2003), and trans-acting short-interfering RNA (ta-siRNA) (Allen et al., 2005; Williams et al., 2005; Hunter et al., 2006), whereas on the abaxial side, KANADI genes (Eshed et al., 2001, 2004; Kerstetter et al., 2001), AUXIN RESPONSE FACTORS (ARF) ETTIN (ETTI/ARF3) and ARF4 (Pekker et al., 2005), YABBY genes (Sawa et al., 1999; Siegfried et al., 1999; Eshed et al., 2004), LITTLE ZIPPER (ZPR) genes (Wenkel et al., 2007), and microRNA165/166 (Bao et al., 2004; Kidner and Martienssen, 2004; Mallory et al., 2004) play important roles.

The HD-ZIPIII gene family consists of members such as REVOLUTA (REV), PHABULOSA (PHB), and PHAVOLUTA (PHV). These genes encode proteins with partially redundant functions. Gain-of-function of one of these genes or simultaneous downregulation of all three genes results in radial cotyledons and leaves (McConnell and Barton, 1998; Emery et al., 2003). Similarly to the HD-ZIPIII gene family, KANADI genes also have overlapping functions. Mutations in any single KAN gene have relatively mild defects in leaf polarity (Eshed et al., 2001; Kerstetter et al., 2001; McAbee et al., 2006). However, if several of these genes are non-functional, the resulting plants exhibit strong defects associated with the loss of abaxial identity. For instance, the kan1 kan2 double mutant has reduced leaf blade expansion and develops ectopic outgrowths on the abaxial side of the...
leaf. More dramatically, in the kan1 kan2 kan3 triple mutant, leaves are almost fully radialized and adaxialized (Eshed et al., 2004).

HD-ZIPIII and KANADI are thought to act antagonistically (Kidner and Timmermans, 2007). The HD-ZIPIII gene PHB is expressed abaxially in kan1 kan2 kan3 triple mutants and its adaxial expression is reduced when KAN2 is expressed throughout the leaf primordia (Eshed et al., 2004). In addition, KAN1 also represses AS2 by directly interacting with its promoter and regulating its transcription (Wu et al., 2008). These results demonstrate that the interactions of key adaxial–abaxial genes are critical for leaf polarity establishment and subsequent blade expansion.

This study identified a new regulator of leaf polarity, APUM23, which interacts with major polarity genes including KANADI, AS2, and REV. APUM23 encodes a protein belonging to the PUF RNA-binding protein family (Abbasi et al., 2010). Drosophila Pumilio is a founding member of this family and is required for the establishment of anterior–posterior polarity (Murata and Wharton, 1995) and stem cell maintenance (Lin and Spradling, 1997; Forbes and Lehmann, 1998) through translation inhibition. PUF proteins specifically bind to nanos response element sequences in the 3′-untranscribed region of target mRNAs (Zamore et al., 1999; Wang et al., 2001; White et al., 2001) and usually function in a complex with other RNA-binding proteins such as Nanos (Sonoda and Wharton, 1999) and Brat (Sonoda and Wharton, 2001). In Arabidopsis, more than 20 putative PUF genes (APUMs) have been predicted by various studies (Francischini and Quaggio, 2009; Abbasi et al., 2010; Tam et al., 2010). Biochemical experiments have shown that several Arabidopsis PUF proteins are able to bind to Drosophila nanos response element sequences and the 3′-untranscribed region of mRNAs from Arabidopsis genes involved in shoot stem cell maintenance, such as WUSCHEL and CLAVATA1 (Francischini and Quaggio, 2009), suggesting a evolutionarily conserved mechanism of PUF protein action. However, in contrast to the products of other APUM genes, APUM23 was found to be localized to the nucleolus and involved in pre-rRNA processing and rRNA maturation (Abbasi et al., 2010). This distinct function of APUM23 is probably due to its unique structure in the PUF RNA-binding domains (Tam et al., 2010).

APUM23 has been shown to play important roles in various aspects of plant growth (Abbasi et al., 2010). The current work shows that APUM23 has a previously undescribed role in regulating the activity of division-competent cells and the establishment of organ polarity. The results suggest that APUM23 is important for organ growth and pattern formation in Arabidopsis.

Materials and methods

Plant materials

The apum23-3, kan1-11, and kan2-5 plants used in this study were in the Columbia (Col) background. as1-1 (CS3774, in Col background), as2-2 (CS3118, in ER background), and rev-1 (CS3826, in No background) were obtained from the Arabidopsis Biology Resource Center (ABRC). The kan1-11 and kan2-5 alleles have been described previously (Wu et al., 2008). All plants were grown at 22 °C under long-day conditions (16/8 light/dark). Double mutants apum23-3 as1-1, apum23-3 as2-2, and apum23-3 rev-1 were generated by initially crossing two parental lines followed by backcrossing the F1 with both parents. The progeny of the backcross was selected for parental phenotypes. The selected plants were self-pollinated and the double mutants were identified from the next generation. F2 progeny from the self-pollinated F1 plants was also examined. The double mutants were found at the ratio of 1/16 or less, and no enhancement of double mutant phenotypes (or other novel phenotypes) was identified in the F2 plants, ruling out background effects on the phenotypes of the double mutants. In order to make the apum23-3 kan1-11 kan2-5 triple mutant, apum23-3 was first pollinated with kan1-11 kan2-5. The F1 then was backcrossed to kan1-11 kan2-5. In the progeny of the backcross, plants homozygous for kan1-11 and apum23-3 and heterozygous for kan2-5 were maintained, and the triple mutants were selected in their progeny by genotyping. The names of the double and triple mutants are abbreviated as kan1 kan2, apum23 as1, apum23 as2, apum23 rev, and apum23 kan1 kan2.

Anatomical analysis

Petioles of 21-d-old plants were fixed in 2% glutaraldehyde in 25 mM sodium phosphate buffer (pH 6.8) with 0.1% Triton X-100 overnight at 4°C. Samples were washed in the same sodium phosphate buffer, dehydrated through an ethanol series, and embedded in wax blocks. Sections (8 μm) were made on a Jung Biocut microtome and stained with 0.1% toluidine blue.

Molecular identification of the enhancer of kan1 kan2

The enhancer of kan1 kan2 mutant was identified by a map-based approach. The single mutants were first crossed with Ler plants. The F2 generation segregating the enhancer phenotype was used as the mapping population, in which 929 mutant plants were identified. Genomic DNA was isolated from these mutants and utilized for mapping by using InDel and cleaved-amplified polymorphic sequence (CAPS) markers based on the Cereon L er/Col SNP database (TAIR; http://www.arabidopsis.org). These molecular markers localized the mutation at the bottom of chromosome I in an approximate 17-kb region containing 13 genes. Sequencing six candidate genes revealed a G→A change that disrupts the intron splicing in At1g72320 (APUM23). In order to confirm the molecular identity of the enhancer as an allele of APUM23, a 6.7-kb genomic DNA fragment containing the entire At1g72320 gene was released from the BAC T10D10 by restriction digestion with SalI and Acc65I (Fermentas, Hanover, MD, USA) and cloned in the binary vector pCAMBIA1300 and transformed into the enhancer mutant using the floral dip method (Clough and Bent, 1998). A control transformation was also performed with the empty pCAMBIA1300 vector. The T1 transgenic plants were selected on 0.1% toluidine blue.

Genetic complementation in yeast

Yeast. Magic Marker npr9 strain (BY4743) was obtained as a heterozygous diploid knock out (open biosystems, Huntsville, AL, USA). APUM23 full-length cDNA was amplified from the BAC clone U09300 (obtained from ABRC with primers GATEARW1N.f (5′-ATGGTTTCTGTTGGTTCTAAATCATTG-3′) and GATEARW1N.r (5′-ACGTCTCAATTCTTATTTTATTTTGAGCAGC-3′). The PCR product was cloned into the pCR8 vector (Invitrogen, Eugene, OR, USA) and subcloned into the yeast expression vector BG1805 (a gift from Beth Grayhack) containing the GAL1
promoter and URA3 marker by GATEWAY cloning strategy (Invitrogen). The resulting plasmid (BG1805-APUM23), as well as the wild-type NOP9 in the same vector (BG1805-NOP9), which was purchased (open biosystems), was transformed into the nop9/+ diploid strain. An empty BG1805 vector was also transformed separately as a control. The transformed cells were sporulated and spread on Magic Medium (Pan et al., 2004) to select haploid cells that survived in the absence of uracil. The transformants were further confirmed by several sets of primers: NOP9F (5′-GTITTTCAACTTTGCTGGTCTGTC-3′) and NOP9B (5′-CCAGTTTCTTCTTACAATGACC-3′) were used to confirm the knock out of NOP9 gene in the nop9 genome, whereas NOP9A and kanB (5′-CTGAGCGAGGGCCGTAAAT-3′) were used to detect the kanMX insertion that knocks out NOP9. Primers BNOVF.1 (5′-CAACCTCAAATGAAATGATACAA-3′) and BNOVR.1 (5′-agactctctggttctgctc-3′) confirmed the presence of BG1805-NOP9 in the haploid cells and BARW1.f (5′-GCCGAAAGCAGTATTTGATTC-3′) and BARW1.r (5′-CCCTCCACACCTCCCTTATTCCT-3′) detected BG1805-APUM23 in the haploid cells. Haploid cells containing either NOP9 or APUM23 were inoculated in the liquid Magic Medium and grown in 30 °C until OD600 reached 0.6. The culture was serially diluted in sterile water and spotted on solid Magic Medium. The plates were incubated at 30 °C for 2 d for analysis.

**Results**

**Identification of an enhancer of the kan1 kan2 leaf polarity phenotype**

An EMS mutagenesis screen was carried out to identify enhancers of the kan1-11 kan2-5 (abbreviated as kan1 kan2) double mutant. kan1 kan2 plants have upward-curving leaves with ectopic outgrowths on the abaxial side (Eshed et al., 2001; Fig. 1C–I). One enhancer mutation was identified that enhanced kan1 kan2 by having rosette leaves with reduced blade expansion (Fig. 1D–J). This defect became more severe as leaf number increased. For instance, leaf 9 of the triple mutant was almost completely radialized indicating a dramatic loss of leaf polarity (Fig. 1J). In addition, the abaxial outgrowths of the kan1 kan2 mutant were also reduced in the triple mutant.

In order to further characterize the mutant phenotypes, the internal structure of the leaf petioles was analyzed. Distinct from the relatively normal midveins of kan1 kan2 double mutant (Fig. 1M), transverse sections showed that the triple mutant had a disorganized vascular pattern consisting of very few phloem cells. These phloem cells were surrounded by adjacent xylem tissues suggesting a strong loss of abaxial identity (Fig. 1N, O). This enhancer is a recessive mutation and the single mutant had elliptical, flat and serrated leaves with a greyish green adaxial side (Fig. 1A, B; see also Fig. 6A). In contrast to the phenotype seen in the triple mutant, the enhancer mutant had very subtle vascular defects with a midvein structure similar to that observed in the wild type Col (Fig. 1K, L).

**The kan1 kan2 enhancer is a mutant allele of APUM23**

The enhancer mutation was mapped to a region containing 13 genes at the bottom of chromosome I, corresponding to the overlap of bacterial artificial chromosomes T10D10 and T9N14. Sequencing of six genes in this region in the enhancer mutant background revealed a single nucleotide mutation (G→A) in APUM23 (At1g72320; Abbasi et al., 2010) that disrupted the 3′-splice site of intron 5 in this gene (Fig. 2A). This suggested that this lesion may account for the enhancer phenotype. The molecular identity of the enhancer was verified by transgene complementation. A 6.7-kb genomic DNA fragment including the wild-type APUM23 was transformed into the enhancer mutant line and resulted in transgenic plants with a wild-type phenotype, while plants carrying the empty vector did not show complementation (Fig. 2B). Moreover, T-DNA insertion alleles of APUM23 also showed similar phenotypes to those of the enhancer (Abbasi et al., 2010; Supplementary Fig. S1 available at JXB online), which further confirmed that the kan1 kan2 enhancer mutation corresponds to a new allele of APUM23, named apum23-3.
apum23-3 interacts synergistically with mutants of genes specifying adaxial identity

In addition to enhancing the kan1 kan2 phenotype, it was also found that apum23-3 showed synergistic interactions with mutants affecting adaxial fate, such as revoluta-1 (rev-1), asymmetric leaves1-1 (as1-1), and asymmetric leaves2-2 (as2-2). REV encodes a member of the HD-ZIP III gene family of transcription factors that redundantly promote adaxial identity (Emery et al., 2003). Due to the overlapping roles of these HD-ZIP III genes, the rev single mutant did not show strong defects in the vegetative stage (Fig. 3A). However, the apum23 rev double mutant had pin-shaped outgrowths in the centre of the rosette that arose after the emergence of several apum23-like, but more elongated leaves (Fig. 3B, C). Petiole sections of the expanded leaves of apum23 rev double mutants displayed abaxialization of vascular tissues with phloem partially surrounding xylem (Fig. 4D), although the vascular pattern of rev-1 was similar to that of the wild type (Fig. 4A). Furthermore, apum23-3 also enhanced the inflorescence defects of rev. The rev mutant inflorescences contained filamentous organs due to the failure of normal floral organ formation (Talbert et al., 1995; Otsuga et al., 2001; Fig 5A, D). The apum23-3 mutant strongly enhanced this defect, such that the double mutant displayed a completely sterile inflorescence with filamentous organs (Fig. 5C, F, G).

As well as the HD-ZIPIII genes, AS1 and AS2 are also key regulators of adaxial fate. AS1 encodes a Myb domain transcription factor closely related to the PHANTASTICA (PHAN) gene product that plays a role in adaxial determination in Antirrhinum (Waites and Hudson, 1995; Waites et al., 1998). AS2 interacts with AS1 (Xu et al., 2006) and participates in establishing leaf polarity by antagonizing KAN genes (Wu et al., 2008). as1 and as2 both produce asymmetric, rumpled leaves with ectopic leaftlet-like structures on petioles.
APUM23 functions in leaf development and polarity

(Tsukaya and Uchimiya, 1997; Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Sun et al., 2002; Fig. 3 D, G). apum23-3 displayed similar interactions with as1 and as2. In the apum23 as1 and apum23 as2 double mutants, only the first four or five leaves were expanded and the subsequent leaves were replaced by pin-shaped or branched structures (Fig. 3F, I). The expanded leaves were also distinct from those of single mutants in that they occasionally produced a ‘trumpet’ shape (Fig. 3E, H). The vasculature of the petioles was also analysed in these mutants. The as1 and as2 mutants often have a slightly abaxialized vein pattern (Ha et al., 2007; Fig. 4B, C). However, in double mutants with apum23, the abaxialization was strikingly enhanced. The midveins of these plants were radialized with phloem surrounding xylem (Fig. 4E, F), which is typical of vascular structures completely lacking adaxial identity.

apum23-3 displays pleotropic defects in development

These strong genetic interactions with key leaf polarity genes imply that APUM23 may play an important role in leaf development. To explore this role, the apum23-3 single-mutant phenotypes were further characterized. The apum23-3 homozygous mutant displayed a variety of developmental defects including delayed leaf formation (2 d later than the wild type, Fig. 6C), delayed phase transition from juvenile to adult as measured by the appearance of the first abaxial trichomes (on leaf 17 in apum23-3 versus leaf 5 in Col; Fig. 6B), and reduced leaf venation complexity (Fig. 6E). The morphology of the subepidermal mesophyll in apum23-3 was also examined as compared to the wild type (Fig. 6D). In wild-type leaves, adaxial mesophyll cells were round and densely packed, whereas abaxial mesophyll consisted of irregular cells with large air spaces. In apum23-3, both adaxial and abaxial mesophyll cells were fewer but slightly bigger than the wild type, and the abaxial cells were more regular in shape. Thus, apum23 affected the differentiation of both adaxial and abaxial cells in the leaf blade. In addition, apum23-3 also had shorter roots (0.9 ± 0.1 cm) as compared to Col (4.1 ± 0.1 cm, Fig. 6F).

apum23-3 affects the expression of leaf polarity genes

APUM23 has been found to be expressed ubiquitously in a variety of organs in Arabidopsis (Abbasi et al., 2010), which implies that it is unlikely to have tissue specific functions in leaf polarity. In order to further examine the effect of APUM23 on leaf polarity genes, the expression of KAN1 and AS2 was assayed in the wild type and apum23-3 mutants using promoter:GUS reporter lines. In wild-type seedlings,
Fig. 3. *apum23-3* interacts with adaxial polarity mutants. Phenotypes of 21-d-old *rev-1* (A), *as1-1* (D), *as2-2* (G), *apum23 rev* (B), *apum23 as1* (E), and *apum23 as2* (H). C, F, and I are higher magnifications of B, E, and H, respectively. White arrowheads in E and H indicate the trumpet-shaped leaves in *apum23 as1* and *apum23 as2*; black arrowheads in C, F, and I show the pin-shaped structures in the double mutants; black arrows in I show the branched radialized structures in *apum23 as2*. Bars, 1 mm (C, F, I) and 1 cm in all other panels (this figure is available in colour at *JXB* online).

Fig. 4. Vascular phenotypes of leaf petioles of adaxial polarity mutants and double mutants with *apum23-3*. Transverse sections of leaf petioles showing vascular organization in 21-d-old *rev-1* (A), *as1-1* (B), *as2-2* (C), *apum23 rev* (D), *apum23 as1* (E), and *apum23 as2* (F) plants. Synergistic interactions between *apum23* and the adaxial mutants are shown by the partial or complete phloem-surrounding-xylem structure in the double mutants. ph, phloem; x, xylem. Bar, 20 μm (this figure is available in colour at *JXB* online).
pKAN1:GUS was expressed in the meristem and the abaxial side of young leaf primordia (Supplementary Fig. S2A available at JXB online), while pAS2:GUS showed a complementary pattern with GUS present only in the adaxial side (Supplementary Fig. S2C available at JXB online). The spatial expression patterns of both GUS reporters were not obviously different in the apum23-3 background (Supplementary Fig. S2B, D available at JXB online), indicating that the spatial expression domains of KAN1 and AS2 are not affected by apum23-3. qRT-PCR was then utilized to measure the mRNA levels of several key leaf polarity genes in the young leaf primordia in the wild type and apum23-3. PHB, REV, AS1, AS2, KAN1, and KAN2 were all expressed at higher level in apum23-3 than in the wild type; however, the expression of TAS3 (ta-siRNA), ARF3, ARF4, and FILAMENTOUS FLOWER (FIL, a major member in the YABBY gene family) were not significantly altered in apum23-3 as compared to those in the wild type (Fig. 7). These results indicated that loss of APUM23 can affect the transcript levels of both adaxial and abaxial regulatory genes, which may be associated with the synergistic phenotypes observed in these double and triple mutant analyses.

Apum23-3 displays defective cell division patterns in both leaves and roots

The apum23-3 mutant had fewer cells in the mesophyll and shorter roots, suggesting a reduction in cell proliferation. To test this idea, the pattern of cell division was examined in both the wild type and apum23-3 using the CYCB1:db:GUS reporter gene (Harrar et al., 2003). GUS staining showed that the number of division-competent cells was reduced in both leaves and roots of apum23-3 as compared to the wild type (Fig. 8). The putative function of APUM23 in promoting cell division is consistent with its strong expression in developmentally active tissues (Abbasi et al., 2010) and is likely correlated with the growth defects seen in apum23 mutants (Fig. 6).

APUM23 functions in pre-rRNA processing

APUM23 belongs to the Pumilio/PUF gene family that is evolutionarily conserved across kingdoms (Spassov and Jurecic, 2003; Abbasi et al., 2010). The APUM23 protein has been found to play a specific role in the cleavage of 35S pre-rRNA, a critical step for 18S rRNA biosynthesis (Abbasi et al., 2010). This function is similar to that of NOP9, a PUF gene in Saccharomyces cerevisiae (Thomson et al., 2007; Abbasi et al., 2010). In order to further confirm the functional homology of APUM23 and NOP9, a complementation experiment was performed in yeast cells. Because the null nop9 mutant is lethal, a heterozygous diploid strain, nop9/+ , was transformed with NOP9 or APUM23 full-length cDNA, and Magic Marker technology (Pan et al., 2004) was utilized to select the nop9 haploid cells carrying NOP9 or APUM23 after sporulation. Growth tests showed that APUM23 could partially rescue nop9 defects (Fig. 9A, B), which suggested some level of functional similarity between these two proteins.
Because the cleavage of 35S pre-rRNA is conserved in eukaryotes (Venema and Tollervey, 1999) and the cleavage site in Arabidopsis has been identified in previous studies (Saez-Vasquez et al., 2004; Shi et al., 2005), the accumulation of the unprocessed 35S rRNA was examined in the wild type and apum23-3 using established methods (Petricka and Nelson, 2007). As expected, the amount of unprocessed 35S rRNA was 2.1-fold higher in apum23-3 compared to the wild type (Fig. 9C). These results further support and extend the observation that APUM23 plays a critical role in 35S pre-rRNA processing (Abbasi et al., 2010).
Discussion

APUM23 is a unique PUF protein involved in rRNA biosynthesis and functions in regulating cell division in Arabidopsis

The *Arabidopsis* genome encodes more than 20 putative PUF proteins (Francischini and Quaggio, 2009; Abbasi et al., 2010; Tam et al., 2010). Among all these PUF family members, APUM23 is the only one that possesses PUF repeats outside the conserved C-terminal PUM-HD region (Tam et al., 2010). Consistent with this unusual structural feature, the major molecular function of APUM23 in regulating 35S pre-rRNA processing (Abbasi et al., 2010; this study) is also distinct from those of other PUF genes in *Arabidopsis* (Francischini and Quaggio, 2009).

Other *Arabidopsis* mutants disrupting rRNA synthesis also have morphological and developmental defects similar to the phenotypes of *apum23* alleles (Abbasi et al., 2010; this study), such as narrow and pointed leaves, defective vein patterning, and reduced root growth and leaf initiation (Shi et al., 2005; Kojima et al., 2007; Petricka and Nelson, 2007). These growth defects have been proposed to be associated with impaired cell division in plants (Petricka and Nelson, 2007). In the current study, the cell division patterns in *apum23* mutants were characterized using *CYCB1:db:GUS* and the results showed that cell division activity is indeed reduced in both roots and leaves of *apum23-3* (Fig. 8). This reduction in cell division is likely associated with the lower number of mesophyll cells in the leaf blade, which results in the pale green colour of the mutant leaves (Fig. 6). These mesophyll cells are also slightly bigger than those of the wild type probably due to ‘compensation’ mechanisms that coordinate cell number and cell size in an organ (Ferjani et al., 2007; Fig. 6). Reduced cell division is also possibly related to the narrow and flat leaves in *apum23-3*, because growth repression results in leaves with reduced curvature and blade expansion (White, 2006). In addition, the short root phenotype is also likely attributable to the reduced activity of cell division zone (Fig. 6). Defective cell division seems to be tightly correlated with the major growth abnormalities observed in the *apum23-3* mutant, so clarifying the genetic mechanisms that account for the reduction in cell division may help lead to a better understanding of the regulation of organ growth by APUM23.

Role of APUM23 in the regulation of leaf polarity

The *apum23*-3 mutant on its own displayed a mild leaf polarity defect. There was a modestly decreased distinction between the adaxial and abaxial mesophyll (Fig. 6), but vascular structures did not differ noticeably from the wild type (Fig. 1). However, mRNAs of a number of polarity genes (*PHB, REV, AS1, AS2, KAN1, KAN2*) accumulated to a higher level in the *apum23* mutants than in the wild type during early stages of leaf development (Fig. 7), which suggests that APUM23 does influence the expression of these critical leaf polarity regulators. Normal leaf polarity is highly dependent on the balance between antagonistic adaxial- and abaxial-specifying genes. In the *apum23* mutant, this balance may not be strongly altered because both types of key regulators are simultaneously upregulated, so the defects in leaf polarity are relatively mild. However, if one or more of these key genes are also mutated, the balance will be altered and proper polarity formation may be disrupted to an even greater degree.

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**Fig. 9.** APUM23 functions in 18S rRNA biosynthesis. (A) Colonies of the haploid *nop9* mutant transformed with empty vector BG1805, *NOP9*, and APUM23, as indicated. (B) Comparisons of a dilution series of haploid *nop9* mutant cells transformed with *NOP9* (upper) and APUM23 (lower), plated on Magic Medium. Cells with APUM23 form smaller colonies than those with NOP9. Fold-dilution is indicated above each column. (C) Enrichment of unprocessed 35S pre-RNA in *apum23*-3 is revealed by semiquantitative reverse-transcription PCR. Error bar represents the standard error of the mean. *UBC1* serves as the loading control.
which may explain the strong polarity defects observed in double and triple mutants with apum23.

The major molecular function of APUM23 is to regulate rRNA biosynthesis, which acts primarily on the translational machinery (Abbasi et al., 2010; this study), but how APUM23 controls the transcription of leaf polarity genes is still not understood. One plausible mechanism is that APUM23 may act indirectly on leaf polarity genes through translational regulation of genes that, in turn, control the transcription of leaf polarity genes. Alternatively, it may be possible that APUM23 also regulates the biogenesis of other RNA species, such as small RNAs, required for leaf polarity. It has been reported that genes involved in rRNA processing are also required for miRNA biogenesis (Fukuda et al., 2007). In leaf polarity, miRNA play important roles to posttranscriptionally regulate critical genes that APUM23 interacts with, such as HD-ZIP III genes (Bao et al., 2004; Juarez et al., 2004; Kidner and Martienssen, 2004; Mallory et al., 2004). Interestingly, HD-ZIP III genes also have similar synergetic interactions with genes encoding subunits of ribosome proteins that interact with rRNA to constitute the ribosome (Pinon et al., 2008; Yao et al., 2008). It has been hypothesized that these ribosomal genes may interact with the small RNA silencing complex RISC to mediate miRNA or siRNA functions, which in turn affect the function of leaf polarity genes (Pinon et al., 2008). Given that the phenotypes of the single mutants of these ribosomal genes and their double mutants with leaf polarity genes both resemble those of apum23 (Pinon et al., 2008; Yao et al., 2008), it is possible that these two essential components of the ribosome may act in related posttranscriptional pathways to control the expression of leaf polarity genes. Future studies to identify the proteins that interact with APUM23 will hopefully provide more insights into this possibility and help to unravel further the function of this unique type of PUF protein in Arabidopsis.

Supplementary material

Supplementary data are available at JXB online.

Supplementary Fig. 1. Phenotype of the confirmed T-DNA insertion line of APUM23 SAIL_757_B08 (apum23-1).

Supplementary Fig. 2. Spatial expression patterns of KAN1 and AS2 in Col and apum23-3.

Supplementary Table S1. Primers used in qRT-PCR.

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