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RESEARCH PAPER

miR396-targeted AtGRF transcription factors are required for coordination of cell division and differentiation during leaf development in *Arabidopsis*

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Abstract

In plants, cell proliferation and polarized cell differentiation along the adaxial–abaxial axis in the primordium is critical for leaf morphogenesis, while the temporal–spatial relationships between these two processes remain largely unexplored. Here, it is reported that microRNA396 (miR396)-targeted *Arabidopsis* growth-regulating factors (AtGRFs) are required for leaf adaxial–abaxial polarity in *Arabidopsis*. Reduction of the expression of *AtGRF* genes by transgenic miR396 overexpression in leaf polarity mutants *asymmetric leaves1* (*as1*) and *as2* resulted in plants with enhanced leaf adaxial–abaxial defects, as a consequence of reduced cell proliferation. Moreover, transgenic miR396 overexpression markedly decreased the cell division activity and the expression of cell cycle-related genes, but resulted in an increased percentage of leaf cells with a higher ploidy level, indicating that miR396 negatively regulates cell proliferation by controlling entry into the mitotic cell cycle. miR396 is mainly expressed in the leaf cells arrested for cell division, coinciding with its roles in cell cycle regulation. These results together suggest that cell division activity mediated by miR396-targeted AtGRFs is important for polarized cell differentiation along the adaxial–abaxial axis during leaf morphogenesis in *Arabidopsis*.

Key words: *Arabidopsis*, AtGRFs, cell differentiation, cell division, leaf polarity, miR396.

Introduction

The development of multicellular organisms relies on the generation of an appropriate number of cells and timely acquisition of specialized cell functions. In flowering plants, leaves are determinate organs, whose development is mediated by the temporal–spatial regulation of cell proliferation and differentiation (Gutierrez, 2005; Ramirez-Parra *et al.*, 2005; Fleming, 2006). Leaf morphogenesis is conceptually divided into three processes: primordia initiation, establishment of polarity, and leaf expansion (Sinha, 1999). After initiation from the peripheral zone of shoot apical meristem (SAM), cells in leaf primordia rapidly divide and are subsequently specified to establish asymmetric growth along the proximo-distal, medio-lateral, and adaxial–abaxial axes (Hudson, 2000; Bowman *et al.*, 2002).

The establishment of the adaxial–abaxial axis is most critical for leaf morphogenesis (Sussex, 1954; Waites and Hudson, 1995; Bowman *et al.*, 2002). In recent years, several families of transcription factor genes have been identified as the key determinants of adaxial or abaxial cell fate. Members of the *KANADI* (*KAN*) and *YABBY* (*YAB*) families and two *AUXIN RESPONSE FACTOR* genes (*ARF3*/*ETT* and *ARF4*) play important roles in determination of abaxial cell fate (Eshed *et al.*, 2004; Pekker *et al.*, 2005). In contrast, three genes in the class III *homeodomain-leucine*

zipper (*HD-ZIP III*) family, namely *PHB*, *PHV*, and *REV*, are expressed in the leaf adaxial domain and redundantly promote adaxial leaf fate (McConnell *et al.*, 2001; Emery *et al.*, 2003). Transcripts of *HD-ZIP III* genes are the targets of microRNA165 and 166 (miR165/166) (Rhoades *et al.*, 2002; Tang *et al.*, 2003), whereas *ARF3/4* are targeted by a trans-acting small interfering RNA (ta-siRNA), termed tasiR-ARF (Allen *et al.*, 2005; Fahlgren *et al.*, 2006). Moreover, the Myb-domain protein ASYMMETRIC LEAVES 1 (AS1) and the LOB domain protein AS2 function together to promote leaf adaxial identity by repressing the abaxial-expressed *KAN* genes and miR165/166 (Byrne *et al.*, 2000; Semiarti *et al.*, 2001; Iwakawa *et al.*, 2002; Kumaran *et al.*, 2002; Xu *et al.*, 2002, 2003; Lin *et al.*, 2003; Li *et al.*, 2005; Wu *et al.*, 2008), while *KAN* genes antagonize the roles of *HD-ZIP III* genes (Emery *et al.*, 2003) and directly repress *AS2* expression by binding to its promoter (Wu *et al.*, 2008). In addition, the 26S proteasome and ribosome proteins were recently implicated to play important roles in promoting the establishment of leaf polarity at the protein regulation level (Huang *et al.*, 2006; Pinon *et al.*, 2008; Yao *et al.*, 2008).

In contrast to the adaxial–abaxial axis, only a few genes were known to be involved in patterning of the proximo-distal (leaf length direction) and medio-lateral (leaf width direction) axes of leaves (Tsukaya, 2006). Growth along the leaf length is governed by *ROTUNDIFOLIA3* (*ROT3*), which regulates polarized cell expansion (Kim *et al.*, 1998), and *ROT4*, which inhibits cell proliferation to regulate specifically cell number in the leaf length direction (Narita *et al.*, 2004). Leaf expansion in the width direction is affected by *ANGUSTIFOLIA* (*AN*) *a*nd *AN3*. *AN* regulates cell expansion in the width direction probably through controlling the cortical microtubule arrangement in leaf cells (Folkers *et al.*, 2002; Kim *et al.*, 2002). *AN3*, which encodes a putative coactivator for the *Arabidopsis* growth-regulating factor (AtGRF) family of transcription factors (J. H. Kim *et al.*, 2003), also called *AtGIF1* (*GRF-interacting factor1*), predominantly controls cell number in the leaf width direction by modulating cell proliferation activity (Horiguchi and Tsukaya, 2003; Kim and Kende, 2004; Horiguchi *et al.*, 2005). The *Arabidopsis AtGRF* family consists of nine members, while *AtGIF1* has another two homologues, *AtGIF2* and *AtGIF3*. Both *AtGRF* and *AtGIF* genes appear to perform redundant functions to promote and/or maintain cell proliferation activity in leaves (J. H. Kim *et al.*, 2003; Kim and Kende, 2004; Horiguchi *et al.*, 2005; Lee *et al.*, 2009), whereas at least seven *AtGRF* genes (*AtGRF1–4* and *AtGRF7–9*) are targeted by miR396, which is encoded by two loci, *MIR396a* and *MIR396b*, and attenuate cell proliferation activity by repression of expression of the targeted *AtGRF* genes (Jones-Rhoades and Bartel, 2004; Liu *et al.*, 2009; Rodriguez *et al.*, 2010).

Although a number of factors have been found to specify leaf polarity, the temporal–spatial relationships of cell proliferation and differentiation during leaf morphogenesis remain largely unexplored. Recently, a novel gene, *AS1/2 ENHANCER7* (*AE7*), which is required for both leaf adaxial–abaxial polarity formation and normal cell proliferation, was characterized. It was also found that the previously characterized 26S proteasome mutant *ae3-1* (Huang *et al.*, 2006) and ribosome mutant *ae5-1* (Yao *et al.*, 2008) exhibited not only leaf adaxial–abaxial polarity but also cell proliferation defects, and it was therefore proposed that normal cell proliferation may be essential for establishment of leaf polarity (Yuan *et al.*, 2010). However, because the 26S proteasome and ribosome are widely recognized as essential machinery for many biological processes, and *AE7* encodes a member of a functionally unknown protein family conserved in eukarytoes, whether this proposal is a general mechanism and how the functions of these proteins in cell proliferation specifically regulate leaf polarity need further investigation.

In this work, it is reported that cell proliferation mediated by miR396-targeted AtGRFs is required for leaf adaxial–abaxial polarity formation during leaf morphogenesis. It is further shown that miR396 negatively regulates cell proliferation in leaves by controlling the entry into the mitotic cell cycle, coincident with its expression in leaf cells arrested for cell division. Because the cells unable to enter into the mitotic cell cycle often undergo enlargement and expansion to start differentiation (Gutierrez, 2005, 2009; De Veylder *et al.*, 2007; Nieuwland *et al.*, 2009), these data together with previous results strongly suggest that active cell division in the primordium is important for leaf adaxial–abaxial polarity formation, and highlight that miR396-targeted AtGRFs may mediate coordination processes of cell division coupled with cell differentiation during leaf morphogenesis.

Materials and methods

Plant materials and growth conditions

The *as1-1*, *as2-1*, and T-DNA insertion mutant *atgif1/an3* (SALK_150407) were obtained from the ABRC. All the plant materials used in this study are in the Columbia-0 (Col-0) background. Plant growth was according to previous conditions (Chen *et al.*, 2000).

Plasmid construction and plant transformation

For transgenic miR396 overexpression, the 316 bp *MIR396A* and *MIR396B* precursor fragments were PCR-amplified with genomic DNA from wild-type Col plants. The DNA fragments were cloned into a modified pCAMBIA2300 vector, behind the duplicated cauliflower mosaic virus 35S promoter, to generate the constructs *35S:miR396a* and *35S:miR396b*. For generation of miR396-resistant versions of *AtGRF9*, the miR396 target site in *AtGRF9* was altered by introducing synonymous mutations using an overlap-PCR method. The modified *AtGRF9* gene was then cloned into a modified pCAMBIA1300 vector, behind the duplicated *35S* promoter. For promoter–GUS (β-glucuronidase) constructs, the 3125 bp and 3132 bp 5′ upstream sequences of *MIR396A* and *MIR396B* were PCR-amplified and cloned into the entry vector pENTR/D-TOPO. The resulting DNA fragments were then recombined into the GATEWAY destination vector pMDC162A (Curtis and Grossniklaus, 2003), to generate *p396a:GUS* and *p396b:GUS* constructs. All constructs were verified by nucleotide sequencing for inserts and introduced into the *Agrobacterium*

strain *GV3101*. The transgenic plants were generated by the floral dip method (Clough and Bent, 1998). The sequences of primers used for PCR are listed in Supplementary Table S1 available at *JXB* online.

Real-time RT-PCR (qRT-PCR)

Total RNAs were extracted from leaves of 13-day-old seedlings using TRIzol reagents (Invitrogen), and reverse transcription was performed with 2 μg of total RNA using a kit (Fermentas, Vilnius, Lithuania). Real-time PCR was performed according to previous methods (Li *et al.*, 2005). Primer sequences are listed in Supplementary Table S1 at *JXB* online.

In situ *hybridization and GUS staining*

In situ hybridizations were performed as previously described (Kim *et al.*, 1998) using 13-day-old seedlings. Detection of miR396 by *in situ* hybridization was performed using the complementary locked nucleic acid (LNA)-modified DNA probe, 5′-AgTTcAAg AAaGCtGGaA-3′, where the lowercase letters represent LNAs. Other probe preparations and GUS staining were according to previous methods (Li *et al.*, 2005). The sequences of primers used for probe preparation are listed in Supplementary Table S1 at *JXB* online.

Microscopy

Scanning electron microscopy (SEM) and preparation of thin-section specimens were according to previous methods (Chen *et al.*, 2000). For differential interference contrast microscopy (DICM) observation, leaves were first fixed in FAA (formalin: acetic acid:70% ethanol, 1:1:8) overnight and then cleared in a chloral solution (chloral hydrate:glycerol:water, 8:2:1) (Tsuge *et al.*, 1996). The determined unit area for cell counting was located in leaves taken at 50% above the base and half way between the midrib and the leaf margin. The unit area was first photographed and then the number of palisade cells within the area was counted.

Flow cytometric analysis

A 0.1 g aliquot of 8-day-old leaves was harvested from 13-day-old seedlings and freshly chopped with a razor blade in a Petri dish containing 2 ml of nuclear isolation and stain buffer (NIM-DAPI 10, Beckman Coulter, CA, USA). The chopped leaves were then filtered through a 40 μm mesh and the isolated nuclei were analysed with a Quanta™ SC flow cytometer (Beckman Coulter). Results from two biological replicates, each with duplicated samples, were analysed.

Results

miR396-targeted AtGRF transcription factors are required for establishment of leaf polarity

To explore how cell proliferation defects affect establishment of leaf polarity, the transgenic plants overexpressing *MIR396a* and *MIR396b* under the control of the cauliflower mosaic virus 35S promoter (*35S:miR396a* and *35S:miR396b*, respectively) were first characterized. Compared with the wild type (Supplementary Fig. S1A at *JXB* online), transgenic *35S:miR396a* (Supplementary Fig. S1B) and *35S:miR396b* (Supplementary Fig. S1C) plants displayed narrow and slightly small rosette leaves, resembling *atgif1/an3* mutants (Horiguchi *et al.*, 2005) (Supplementary Fig. S1D). Furthermore, similarly to *atgif1/an3* (Supplementary Fig. S1H), the size of the palisade mesophyll cells in *35S:miR396a* (Supplementary Fig. S1F) and *35S:miR396b* (Supplementary Fig. S1G) plants was clearly bigger than those of the wild-type (Supplementary Fig. S1E), while the cell numbers in those plants are significantly reduced (Supplementary Fig. S1I). qRT-PCR analysis revealed that the transcription levels of the nine *AtGRF* genes including seven predicated *AtGRF* gene targets in *35S:miR396a* and *35S:miR396b* plants were all decreased (Supplementary Fig. S1J), indicating that miR396 negatively regulates the cell proliferation in leaves by repressing the expression of *AtGRF* genes, consistent with reported results (Liu *et al.*, 2009; Rodriguez *et al.*, 2010).

The roles of miR396-targeted AtGRFs in establishment of leaf polarity were then investigated by introducing the *35S:miR396a* and *35S:miR396b* constructs into the leaf polarity mutants *as1-1* (Fig. 1A) and *as2-1* (Fig. 1E), which exhibit only weak leaf adaxial–abaxial polarity defects (Byrne *et al.*, 2000; Semiarti *et al.*, 2001; Iwakawa *et al.*, 2002; Kumaran *et al.*, 2002; Xu *et al.*, 2002, 2003; Lin *et al.*, 2003). In contrast, the *35S:miR396/as1-1* (Fig. 1B, C) and *35S:miR396/as2-1* (Fig. 1F, G) transgenic plants produced stronger leaf polarity defects; the first two rosette leaves typically appeared lotus like and, in some extreme cases, needle like. In the F_3 progeny of three independent homozygous lines, >80% of *35S:miR396a/as2* plants produced lotus-like or needle-like leaves in the first pair of true leaves, while <5% of *as2* plants generated lotus-like leaves (Fig. 1I), indicating that miR396-AtGRFs and *AS1/2* pathways synergistically regulate leaf adaxial–abaxial formation.

The transcription levels of *AtGRF* genes were analysed in *35S:miR396a/as2* transgenic plants by qRT-PCR. Similar to *35S:miR396*/Col transgenic plants, the expression levels of *AtGRF* genes were decreased in *35S:miR396a/as2* (Fig. 1J), suggesting that the leaf polarity defects in *35S:miR396a/as2* plants were due to the decreased expression of *AtGRF* genes. To support these results, the double mutants *an3 as1* and *an3 as2* were also generated. The phenotype of the *an3 as1* (Fig. 1D) and *an3 as2* (Fig. 1H) double mutants largely resembled that of *35S:miR396/as1* and *35S:miR396/as2* plants, respectively. To confirm this further, the construct *35S:rAtGRF9*, which carries the miR396-resistant version of *AtGRF9* (*rAtGRF9*), was made and introduced into *35S:miR396a/as2* plants. The miR396 target sequence in *AtGRF* genes is located at the end of the region encoding the conserved WRC domain (Fig. 2A). Disruption of the miR396-binding sites in *AtGRF9* could result in the accumulation of their stabilized transcripts in transgenic plants. By qRT-PCR, it was found that the *AtGRF9* mRNA in the double transgenic plants was increased to levels higher than in the wild type, in contrast to the much lower accumulation in *35S:miR396a/as2* plants (Fig. 2D). Accordingly, it was observed that the leaf polarity defects (as indicated by the appearance of lotus-like leaves, Fig. 2C) of *35S:miR396/as2-1* could be rescued by the introduced *rAtGRF9* gene (Fig. 2E, F) but not by an empty vector (Fig. 2B). Taken together, these results suggested that the AtGRF–AtGIF complexes are required for establishment of leaf polarity, and the reduction of *AtGRF* gene expression

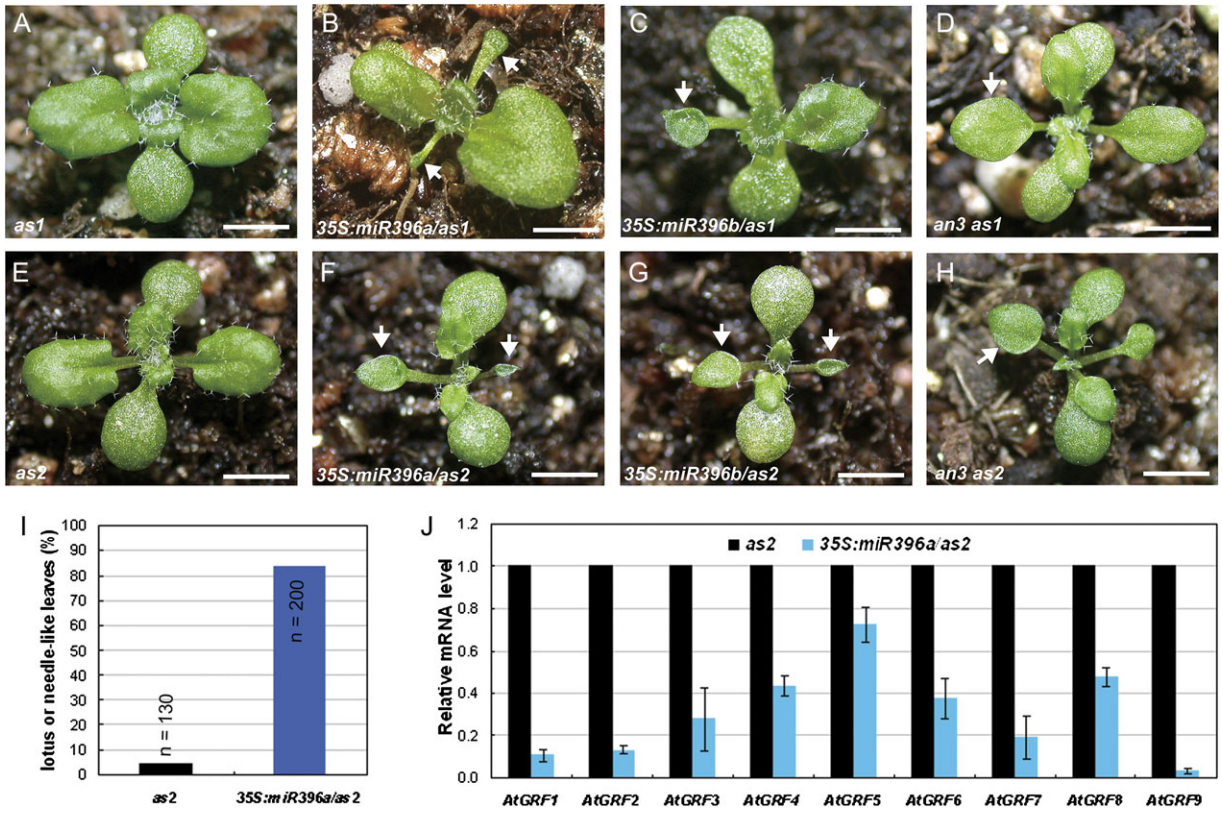

Fig. 1. miR396-targeted AtGRF transcription factors are required for establishment of leaf polarity. (A–H) Morphological observations of the *as1* (A), *35S:miR396a/as1* (B), *35S:miR396b/as1* (C), *an3 as1* (D), *as2* (E), *35S:miR396a/as2* (F), *35S:miR396b/as2* (G), and *an3 as2* (H) plants. Arrowheads in B–D and F–H indicate the lotus- or needle-like leaves. (I) Lotus- or needle-like leaves were present more frequently in *35S:miR396a/as2* plants than in the *as2* mutant. The first pair of rosette leaves was analysed for the presence of lotus- or needle-like leaves. n, numbers of plants analysed; (J) qRT-PCR to analyse the transcription levels of *AtGRF* gens in 14-day-old seedlings of *as2* and *35S:miR396a/as2* plants. Bars=1 cm in A–H.

by transgenic miR396 overexpression resulted in the leaf polarity defects in *35S:miR396a/as2* plants.

Enhanced defects of abaxial–adaxial polarity in the leaves of 35S:miR396a/as2 *plants*

To better understand the roles of miR396 and *AtGRF* genes in leaf patterning, the leaf phenotype of *35S:miR396a/as2* transgenic plants was analysed in more detail by SEM. The adaxial epidermis of both wild-type and *as2* leaves was characterized by an undulating surface comprising uniformly sized pavement cells (Fig. 3A, D), while smaller pavement cells interspersed with some long and narrow cells were noted in the abaxial epidermis (data not shown). The epidermal cell patterns of *35S:miR396* plants were normal (data not shown). However, the adaxial surface of the lotus-like leaves of *35S:miR396a/as2* transgenic plants comprised a mosaic of both adaxial and abaxial epidermal cells, characterized by the presence of abaxialized long and narrow cells in the adaxial surface (Fig. 3B, E). The extremely narrow and needle-like leaves of *35S:miR396a/as2* plants exhibited long and narrow abaxial epidermal cells (Fig. 3C, F), suggesting that this leaf is abaxialized.

The vascular pattern of *35S:miR396a/as2* plants was further analysed by transverse sectioning through the blade–petiole conjugation region. In the wild-type or *as2* leaves, vascular bundles in this region showed a pattern whereby xylem develops on the adaxial pole and phloem is located on the abaxial pole (Fig. 3G). In the *35S:miR396* transgenic plants, the vascular pattern in the expanded rosette leaves did not show obvious changes (data not shown). In contrast, the lotus-like leaves of *35S:miR396a/as2* plants exhibited a phloem-surrounding-xylem structure (Fig. 3H), while the needle-like leaves of *35S:miR396a/as2* plants exhibited no apparent vascular bundle but numerous intercellular spaces (Fig. 3I).

To obtain molecular evidence that miR396-targeted AtGRFs are required for establishment of leaf polarity, the expression pattern of a *YABBY* family gene, *FIL/YAB1*, was examined by *in situ* hybridization. *FIL* is usually expressed on the leaf abaxial domain of both the wild type (Fig. 3J) and the *as2* mutant (Fig. 3K). In contrast, *FIL* was expressed throughout the entire primordium of the needle-like leaves of *35S:miR396a/as2* plants and seemed to accumulate to higher levels in the other leaves (Fig. 3L). These results together indicated that miR396-targeted AtGRFs are required for leaf patterning and

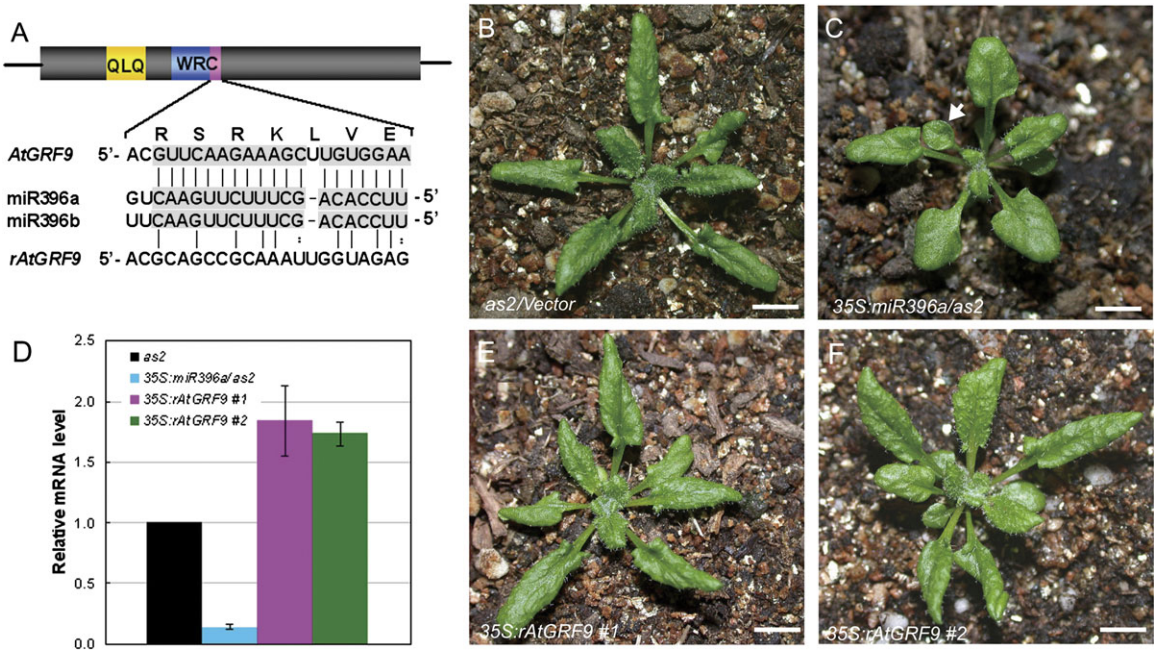

Fig. 2. Introduction of the miR396-resistant *AtGRF9* gene into a *35S:miR396a/as2* plant partially rescued the leaf polarity defects. (A) Diagram of the miR396 target sites of the wild type and a modified version of *AtGRF9*. The conserved QLQ motif in AtGRFs is indicated in yellow, and the WRC motif is shown in blue, with the miR396 target region marked in purple. (B, C) Morphological observation of the 27-day-old seedlings of *as2* (B) and *35S:miR396a/as2* (C). The arrowhead in C indicates the lotus-like leaf. (D) qRT-PCR analysis of the *AtGRF9* transcript levels in *as2*, *35S:miR396a/as2*, and *35S:miR396a/as2* expressing the miR396-resistant *AtGRF9* gene. (E, F) Morphology of the 27-day-old seedlings of *35S:miR396a/as2* expressing the miR396-resistant *AtGRF9* gene driven by the 35S promoter. #1, line1; #2, line2. Bars=1 cm in B, C, E, and F.

genetically regulate the expression of leaf polarity-controlling genes.

miR396 negatively regulates cell proliferation by controlling entry into the mitotic cell cycle

In *Arabidopsis*, the proliferating cells in lateral organ primordium usually divide in the mitotic cell cycle, which follows the common scheme of G_1, S, G2, and M phases (Inze and De Veylder, 2006; Gutierrez, 2009), to multiplify and promote organ growth and development. To gain insights into how the roles of miR396 and AtGRFs in cell proliferation affect establishment of leaf polarity, the expression pattern of one of the cell cycle marker genes, *histone H4*, was analysed by *in situ* hybridization. *Histone H4* is predominantly expressed in the proliferating cells in the DNA replication-related S phase of the cell cycle and thus might mark the cells undergoing mitotic cell division (Reichheld *et al.*, 1995, 1998). Compared with the wild type (Fig. 4A), the number of leaf cells expressing *histone H4* was dramatically reduced in the *35S:miR396a* plants (Fig. 4B, C). In addition to *histone H4*, the expression of several other cell cycle-related marker genes was also analysed by qRT-PCR. *Cyclin D3;1* (*CycD3;1*) is mainly expressed at proliferating cells in the G_1 phase and drives the G_1/S transition together with cyclin-dependent kinase A (CDKA) (Dewitte *et al.*, 2003; Menges *et al.*, 2006). *CYCA2;1* is involved in S/G_2 transition, and *CYCB1;1* in G_2/M transition (Doerner *et al.*, 1996; Shaul *et al.*, 1996). The expression of all the tested marker genes was reduced in *35S:miR396a* plants with a 2- to 4-fold change (Fig. 4D), indicating that the defective cell proliferation in leaves of *35S:miR396* plants could be due to the reduced cell division activity. The expression of cell cycle-related genes was also examined in *as2* and *35S:miR396a/as2* plants. Similarly, the number of cells expressing *histone H4* was reduced (Supplementary Fig. S2B, C at *JXB* online), and the mRNA levels of cell cycle marker genes were reduced even more (5- to 15-fold change) in *35S:miR396a/as2* plants (Fig. S2D) as compared with those in the *as2* mutant (Fig. S2A, D), indicating that the cell division activity mediated by miR396-targeted AtGRFs is important for establishment of leaf adaxial–abaxial polarity.

To dissect further the phase of the cell cycle affected by transgenic miR396 overexpression, a flow cytometric analysis was performed with nuclei from leaves of ~8 d old to examine the nuclear ploidy level. The *atgif1/an3* leaves were analysed as a control. The percentages of 8C and 16C cells in *35S:miR396* and *atgif1/an3* plants were significantly higher than those in the wild-type plants, whereas the percentages of 2C and 4C cells were reduced accordingly (Fig. 4E, F). Because it has been proposed that at this time point leaf cells have the capacity to divide and 2C and 4C cells could represent the G_1 and G_2 cells, respectively (Takahashi *et al.*, 2008), the ratio of 4C/2C cells in 8-day-old leaves was compared further. As shown in Fig. 4G,

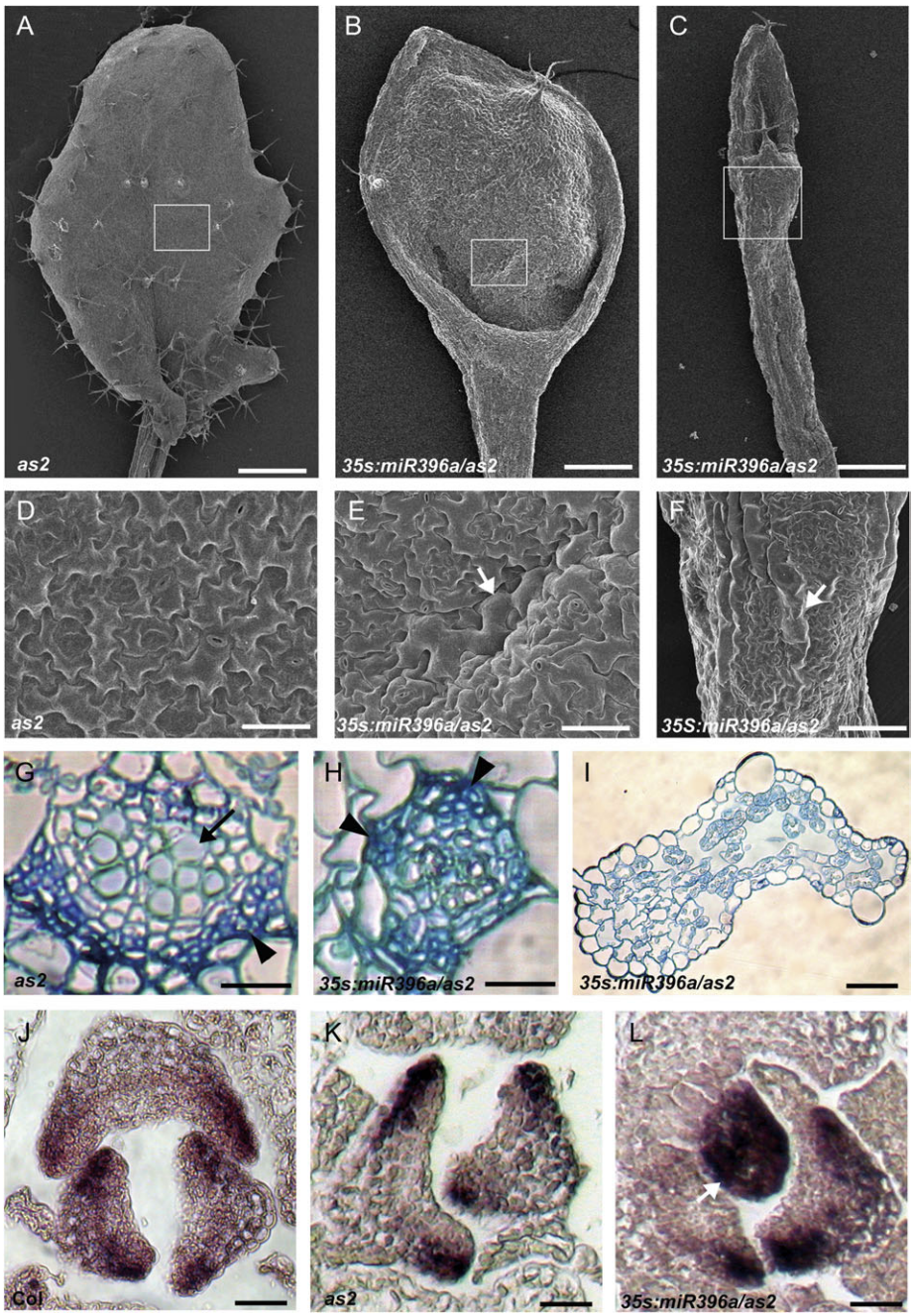

Fig. 3. Enhanced adaxial–abaxial defects of leaf polarity by transgenic miR396 overexpression in the *as2* mutant. (A–C) Comparison of the first rosette leaf of *as2* (A) with the lotus-like (B) and needle-like (C) leaves of *35S:miR396a/as2*. (D–F) Epidermal cells on the adaxial surface of *as2* (D), *35S:miR396a/as2* lotus-like (E) and needle-like (F) leaves. Arrowheads in E and F indicate the long and narrow abaxialized-featured epidermal cells. (G–I) Transverse sections through the blade–petiole junction region showed vascular patterns of leaves in *as2* (G), *35S:miR396a/as2* lotus-like (H) and needle-like (I) leaves. Arrows and arrowheads in G and H show xylem and phloem, respectively. (J–L) *In situ* hybridization to analyse the *FIL* transcripts in Col (J), *as2* (K), and *35S:miR396a/as2* (L) seedlings. The arrowhead in L indicates the leaf primordium uniformly expressing *FIL*. Bars=1 mm in A, 500 μm in B and C, 50 μm in D and G–I, and 100 μm in E, F, and J–L.

the ratio of 4C/2C cells was slightly decreased in *35S:miR396* and *atgif1/an3* leaves compared with those of the wild type.

It is known that in response to a variety of physiological signals or developmental cues, proliferating cells can exit from the mitotic cell cycle to an alternative cycle, the endoreplication cycle (endocycle), for expansion/differentiation, whereby cells replicate the full genome to increase the ploidy level (from 2C to 2^nC) but fail to undergo neclear division to produce progeny cells (De Veylder *et al.*, 2007; Gutierrez, 2009). The switch to the endocycle also requires that, at least, *CYCD3;1* expression is turned off (Dewitte and Murray,

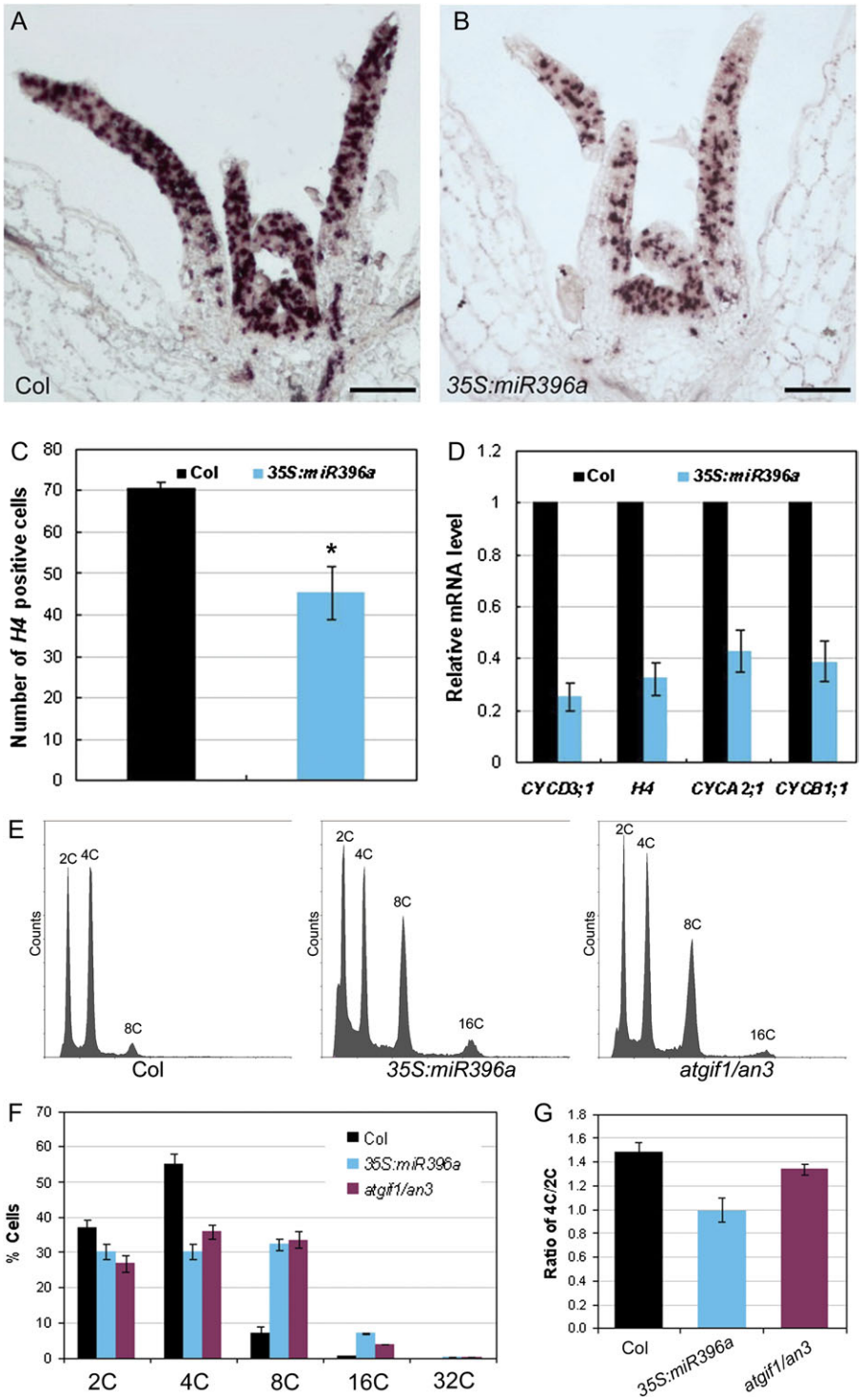

Fig. 4. miR396 negatively regulates cell proliferation by controlling entry into the mitotic cell cycle. (A and B) *In situ* hybridization analysis of *histone H4* expression in shoot apices of 13-day-old wild-type (A) and *35S:miR396a* (B) seedlings. (C) The number of *histone H4*-positive cells in leaves of the 13-day-old wild-type and *35S:miR396a* seedlings. The asterisk indicates a statistically significant difference by *t*-test ($P < 0.05$). (D) Expression of cell cycle-related genes in 13-day-old wild-type and *35S:miR396a* seedlings. (E–G) Ploidy level distribution analysis. The ~8-day-old leaves from 13-day-old seedling of the wild type, *35S:miR396a*, and *atgif1/an3* were used to examine the cell nuclear ploidy levels (E). The percentages of cells with different nuclear ploidy levels (F) and the ratio of 4C/2C cells (G) were calculated. Bars=100 μm in A and B.

2003). Because both the number of cells undergoing mitotic cell division and the cell cycle marker genes including *CYCD3;1* were reduced, but the percentage of leaf cells with higher ploidy levels was increased in *35S:miR396* plants, these results indicate that miR396 appears to play important roles in controlling the entry into the mitotic cell cycle.

miR396 is mainly expressed in leaf cells arrested for cell division

To analyse the temporal–spatial regulation of cell division by miR396–AtGRF modules, the expression patterns of *AtGRF2*, *AtGRF9*, and *AtGIF1/AN3* were first examined by *in situ* hybridization. *AtGRF2* and *AtGRF9* were expressed in the SAM and young leaf primordium (Fig. 5A, B). Along the adaxial–abaxial axis, they were expressed uniformly throughout the young leaf primordia, similar to the pattern of *AS1* (Fig. 5D), which is believed to be expressed in the whole primordium (Byrne *et al.*, 2000). Interestingly, the transcripts of *AtGIF1/AN3* appeared to be highly accumulated in the proximal part of leaf primordia (Fig. 5C), in which cells are rapidly proliferating, consistent with the previous results obtained by promoter–GUS assay (Horiguchi *et al.*, 2005).

The expression pattern of miR396 was further investigated using an antisense LNA-modified DNA probe, which could detect the mature products of both *MIR396a* and *MIR396b*. As shown in Fig. 5E, the LNA signals were detected in the SAM and leaf primordia, and appeared to have a higher level in the distal part of primoridia, but no

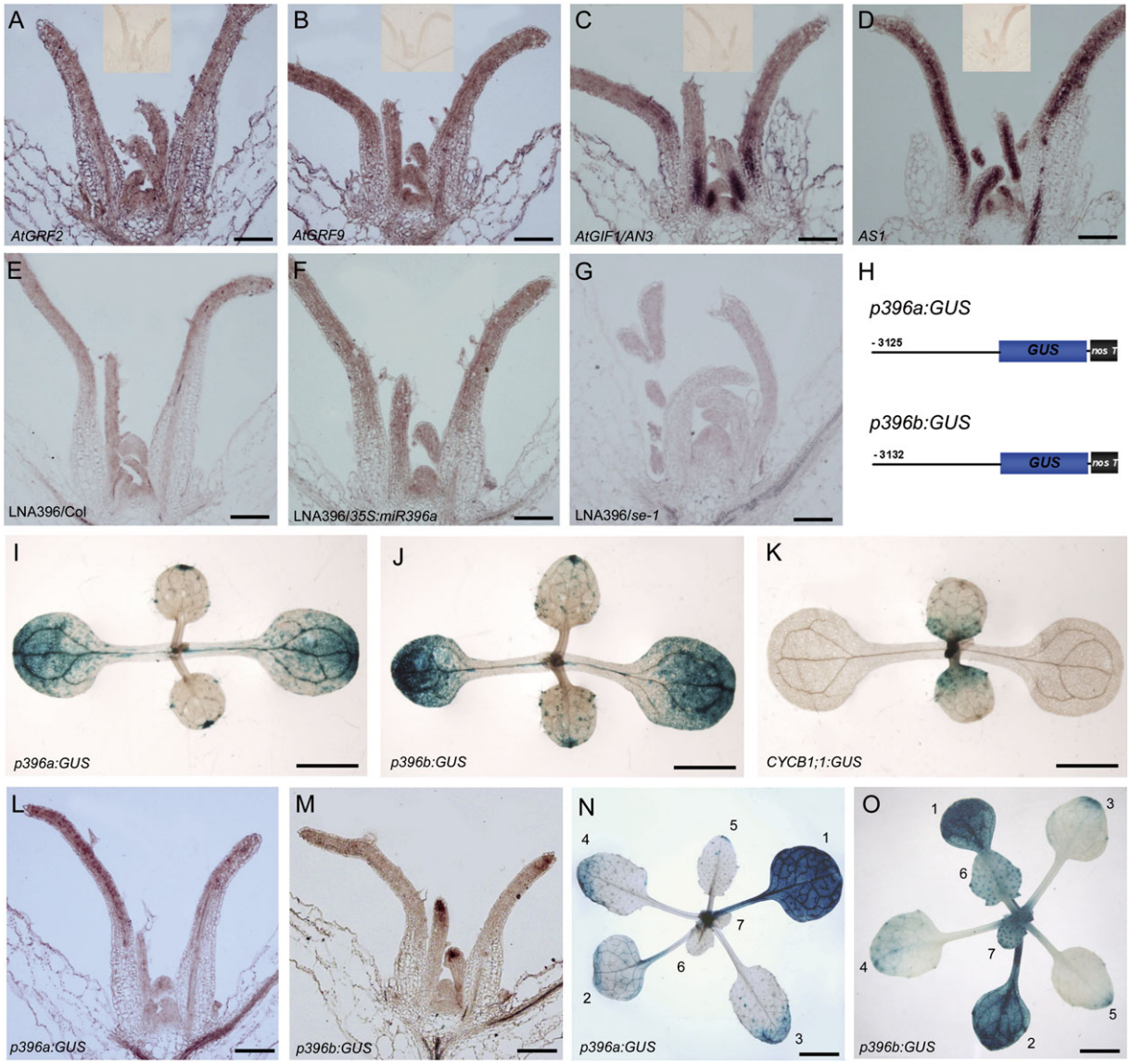

Fig. 5. miR396 is mainly expressed in the cells arrested for cell division. (A–D) *In situ* hybridization analysis of *AtGRF2*, *AtGRF9*, *AN3*, and *AS1* expression in shoot apices. The insets show no signals detected by the sense probes of the corresponding genes. (E–G) The antisense LNA probe detected miR396 in the shoot apex of wild-type (E), *35S:miR396a* (F), and *se-1* (G) seedlings. (H) Diagram of the structures of *p396a:GUS* and *p396b:GUS* constructs. (I–K) GUS staining to analyse the GUS distribution patterns in the 9-day-old *p396a:GUS* (I), *p396b:GUS* (J), and *CYCB1;1:GUS* (K) seedlings. (L and M) *In situ* hybridization to analyse the GUS distribution pattern in the *p396a:GUS* (L) and *p396b:GUS* (M) seedlings, respectively. (N and O) GUS staining to analyse the GUS distribution pattern in the 24-day-old seedlings of *p396a:GUS* (N) and *p396b:GUS* (O). The numbers 1–7 indicate the number of leaves. Bars=100 μm in A–G, L, and M, 0.5 cm in I–K, N, and O.

apparent polar distribution along the adaxial–abaxial axis was observed. The specificity of the hybridization signal was confirmed using the seedlings of *35S:miR396a* transgenic plants (Fig. 5F; high miR396 level) and *se-1* (Fig. 5G; low miR396 level), in which the LNA probe detected strong signals or almost no signals, respectively. Due to the relatively low accumulation of miR396 revealed by LNA *in situ* hybridization, the functional domain of the promoters of *MIR396A* and *MIR396B* in the leaves was therefore characterized to better understand the expression pattern of miR396. The ~3.2 kb 5' upstream regions of *MIR396A* and *MIR396B* (counting from the first nucleotide of the mature miR396 sequences) were cloned to represent their respectiveputative promoters. The fragments were fused with the *GUS* reporter gene to generate the *p396a:GUS* and *p396b:GUS* constructs (Fig. 5H), which were then introduced into wild-type plants. By examining the GUS staining signals, it was found that dynamic but similar patterns for both constructs were detected from the cotyledon, shoot apex, and rosette leaves (Fig. 5I, J, L–O). *In situ* hybridization with a *GUS*-specific probe revealed that the GUS signals were distributed in both the adaxial and abaxial domains (Fig. 5L, M), suggesting that miR396 may not have a polar distribution along the adaxial–abaxial axis in leaves. In contrast, the GUS staining signals appeared to be dynamically distributed along the proximal–distal axis of leaves and varied during leaf development. In the cotyledon, the GUS signals were very strong (Fig. 5I–J), in contrast to almost no signals of *CYCB1;1:GUS* (Colon-Carmona *et al.*, 1999) in the cotyledon. In young leaves, the GUS signals were strong in the tip and distal parts, opposite to the expression pattern of *CYCB1;1:GUS*. However, when the leaves gradually become mature, the GUS signals progressively accumulate from the distal to the proximal part of developing leaves, with strong activity in the whole mature leaves (Fig. 5N, O). It should be pointed out that the expression patterns of miR396 revealed by promoter–GUS assay do not completely coincide with those by LNA *in situ* hybridization analysis. This may be due to the non-cell-autonomous movement of mature miR396 to adjacent cells from the original biosynthetic cells. Because the leaf primordia emerging from the SAM first undergo proliferative cell division, and subsequently the progression of cell division arrest in leaves moves from the distal leaf tip to the proximal leaf part in *Arabidopsis* (Donnelly *et al.*, 1999), the results together indicate that miR396 is mainly expressed in the leaf cells arrested for the cell cycle in the distal part of either developing leaves or mature leaves.

Effects of miR396 misexpression on the establishment of leaf adaxial–abaxial polarity

To investigate how the temporal–spatial regulation of cell division by miR396 affects the establishment of leaf polarity, the effects of miR396 expression driven by tissue-specific promoters in different shoot apex domains of the *as2* mutant on establishment of leaf polarity was analysed. The promoter of *SHOOT MERISTEMLESS* (*STM*) was used for expression in the SAM (J. Y. Kim *et al.*, 2003), those of *AS1* (Byrne *et al.*, 2000) and *AN3* (Horiguchi *et al.*, 2005) (Fig. 5C) for young leaf primordia, that of *REV* for the adaxial domain of young leaf primordia and the central domain of SAM (Emery *et al.*, 2003; Otsuga *et al.*, 2001; Prigge *et al.*, 2005), and that of *FIL* for the abaxial domain of young leaf primordia (Siegfried *et al.*, 1999; Watanabe and Okada, 2003). All the constructs were introduced into the *as2* mutant background and >20 independent transgenic plants were analysed for each construct. As shown in Fig. 6, it was found that the tissue-specific reduction of cell division in leaf primordia by *pAS1:miR396a* (Fig. 6B) and *pAN3:miR396a* expression (Fig. 6C) in the *as2* mutant (Fig. 6A) showed enhanced leaf polarity defects in transgenic plants, as indicated by the presence of lotus- or needle-like leaves, indicating that cell division in the rapidly proliferating

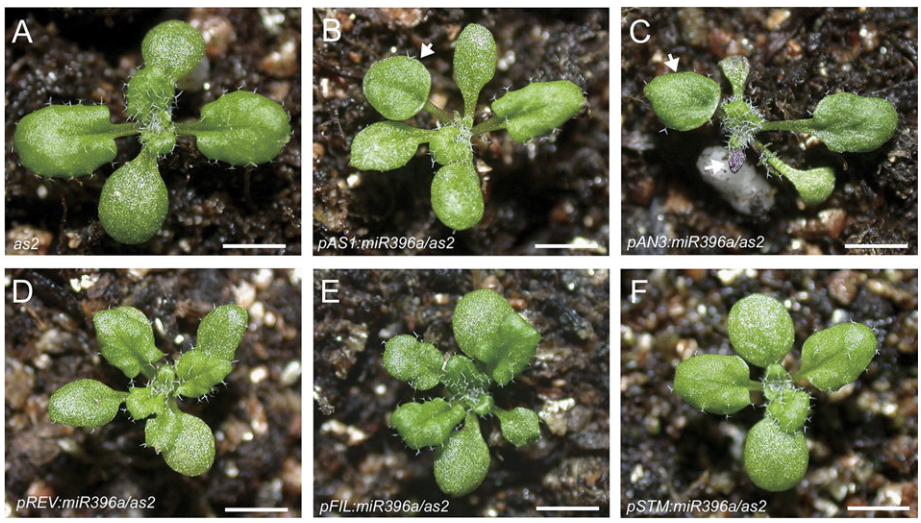

Fig. 6. Effects on establishment of leaf polarity of miR396 misexpression in the *as2* mutant. (A–F) Morphology of the 16-day-old seedlings of *as2* (A) and *as2* expressing *pAS1:miR396a* (B), *pAN3:miR396a* (C), *pREV:miR396a* (D), *pFIL:miR396a* (E), and *pSTM:miR396a* (F). Bars=1 cm in A–F.

primordia is more important for establishment of leaf polarity. Typically, the leaf polarity defects in the *pAN3:miR396a/as2* transgenic plants were stronger than those of *pAS1:miR396a/as2* plants, which may reflect the consequence of their different expression domain in leaf primordia. Transgenic miR396 expression driven by the promoter of *REV*, *FIL*, and *STM* in *as2* mutants failed to enhance the polarity defects of *as2*, except for the slightly small and dwarf plant architecture (Fig. 6D–F), suggesting that either the activities of these three promoters were not strong enough or reduction of cell division in the specific adaxial/abaxial domain of young leaf primordia and the SAM had minor effects on the establishment of leaf polarity.

Discussion

Previously, computational prediction implied that at least seven *AtGRF* genes contain the target sites of miR396 (Jones-Rhoades and Bartel, 2004), and recent transgenic experiments (Liu *et al.*, 2009; Rodriguez *et al.*, 2010) and the characterization of *35S:miR396a* and *35S:miR396b* transgenic plants in this study (Supplementary Fig. S1 at *JXB* online) all demonstrated that miR396 negatively regulates cell proliferation by repressing *AtGRF* gene expression in *Arabidopsis*. To date, miR396 and its cognate *GRF* targets have been found to be present in >30 plant species from gymnosperms to monocots and eudicots (Axtell and Bartel, 2005; Sunkar and Jagadeeswaran, 2008), indicating that miR396–GRF regulatory modules have an ancient origin and may be important for flowering plant evolution and development.

Cell division, expansion, and differentiation are pivotal processes necessary for organogenesis. A major developmental question is how cells acquire specific fates in conjunction with cell division and integration into developmental processes. During leaf morphogenesis, cell division and differentiation along the adaxial–abaxial axis result in the two leaf faces being distinct in both structure and biological function (McConnell *et al.*, 2001). In this study, the cell division activity mediated by miR396-targeted AtGRFs was found to be required for leaf adaxial–abaxial polarity and trichome formation, revealing tight coordination of cell division and differentiation during leaf morphogenesis. It was observed that transgenic miR396 overexpression in *as1/2* mutants resulted in plants with enhanced leaf adaxial–abaxial polarity, which was correlated with the decreased cell division activity accompanying the decreased expression of *AtGRF* genes (Supplementary Fig. S2 at *JXB* online). The recent characterization of *ae7* and the previously reported 26S proteasome mutant *ae3-1* and ribosome mutant *ae5-1* showed that these three mutations affected both leaf adaxial–abaxial polarity and cell proliferation, and it was therefore proposed that normal cell proliferation may be essential for the establishment of leaf polarity (Yuan *et al.*, 2010. Studies on the roles of miR396–AtGRF modules in the establishment of leaf polarity provide further evidence and extend this proposal, suggesting that normal cell division is coupled with polarized cell proliferation and is important for the establishment of leaf polarity.

It is known that the switch from cell proliferation to differentiation often coincides with the switch from the mitotic cell cycle to the endocycle during leaf development (Boudolf *et al.*, 2004). Although not always, the endoreplication is frequently correlated with an increase in cell size. In leaves, the existence of the compensation syndrome, in which a decrease in cell number triggers an increase in mature cell size, also suggests the tight coordination of cell proliferation and expansion/differentiation (Tsukaya, 2006, 2003). Similarly to *atgif/an3*, the reduction of cell numbers in *35S:miR396* leaves was accompanied by the enlargement of cell size (Supplementary Fig. S1 at *JXB* online) and an increased percentage of 8C and 16C cells, suggesting that miR396 has functions in promoting the entry of cells into the endocycle for expansion/differentiation. Cell differentiation in leaf primordia along the adaxial–abaxial axis (McConnell *et al.*, 2001) usually occurs in a sequential fashion for an ultimately functional leaf, and the abaxial spongy cells stop proliferating and start to differentiate earlier than the tightly arranged adaxial palisade cells (Yuan *et al.*, 2010). Therefore, it is possible that cell proliferation defects caused by transgenic miR396 overexpression in the *as1/2* mutant background may result in cell divison stopping earlier, and thereby early differentiation of abaxial cells and insufficient cells for differentiation into the leaf adaxial domain, which in turn might lead to the premature establishment of abaxial cell fate accompanied by ectopic expression of abaxial-specific genes such as *FIL* in the leaf primordia, because the adaxial and abaxial domains specified by the polarity-related genes are mutually antagonized.

The negative roles of miR396 in cell proliferation appear to be due to the reduced cell division activity. First, the numbers of dividing cells in the leaf primordia of *35S:miR396* were decreased, as revealed by the decreased number of cells expressing *histone H4*. Secondly, the expression of cell cycle-related genes including *CYCD3;1*, *histone H4*, *CYCA2;1*, and *CYCB1;1* was found to be reduced in *35S:miR396* plants. Finally, the percentages of 8C and 16C cells in *35S:miR396a* and *atgif/an3* leaves were significantly increased. All these results indicate that miR396 negatively regulates cell proliferation by controlling entry into the mitotic cell cycle. This is also consistent with the observation that the leaves of *35S:miR396* plants displayed a reduced cell number but enlarged cell size (Supplementary Fig. S1 at *JXB* online), because the cells unable to enter into the mitotic cell cycle could undergo endoreplication to increase the ploidy level and cell size in leaves. Thus, miR396 may have functions to prevent mitotic cell division and thereby promote cell differentiation during leaf development.

Analysis of the expression pattern of miR396 further supported the roles of miR396 in controlling entry into the mitotic cell cycle. By *in situ* hybridization and promoter–GUS assays, miR396 was found to accumulate mainly in the distal part of developing leaves, progressively from the

distal to the proximal part, whereas it appears to accumulate throughout almost the whole cotyledon and mature leaves in cells which have stopped dividing, coinciding with its roles in controlling entry into the mitotic cell cycle. In addition, the putative promoters of both *MIR396A* and *MIR396B* exhibited high activities in the leaf trichomes (Supplementary Fig. S3 at *JXB* online). Leaf trichomes are large single cells originating from the epidermis, in which trichomes are the first epidermal cells that begin to differentiate from the developing leaf primordia (Hulskamp *et al.*, 1994; Larkin *et al.*, 1996). After trichome fate commitment the cells stop dividing and switch to the endocycle, resulting in a DNA content of 32C on average in the mature trichome (Hulskamp *et al.*, 1994; Hulskamp, 2004; Schellmann and Hulskamp, 2005). Thus, the mature miR396 is presumably biosynthesized in the single trichome cells to inhibit their further division, consistent with the role of miR396 in controlling entry into the mitotic cell cycle.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. miR396 negatively regulates cell proliferation by repressing the expression of *AtGRF* genes in the leaves.

Figure S2. *In situ* hybridization and qRT-PCR analyses of cell cycle-related genes in *as2* and *35S:miR396a/as2* transgenic plants.

Figure S3. Expression of *p396a:GUS* and *p396b:GUS* in leaf trichomes.

Table S1. The sequences of the primers used in this study.

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