

Control of cell proliferation in *Arabidopsis thaliana* by microRNA miR396

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SUMMARY

Cell proliferation is an important determinant of plant form, but little is known about how developmental programs control cell division. Here, we describe the role of microRNA miR396 in the coordination of cell proliferation in *Arabidopsis* leaves. In leaf primordia, miR396 is expressed at low levels that steadily increase during organ development. We found that miR396 antagonizes the expression pattern of its targets, the *GROWTH-REGULATING FACTOR* (*GRF*) transcription factors. miR396 accumulates preferentially in the distal part of young developing leaves, restricting the expression of *GRF2* to the proximal part of the organ. This, in turn, coincides with the activity of the cell proliferation marker *CYCLINB1;1*. We show that miR396 attenuates cell proliferation in developing leaves, through the repression of *GRF* activity and a decrease in the expression of cell cycle genes. We observed that the balance between miR396 and the *GRFs* controls the final number of cells in leaves. Furthermore, overexpression of miR396 in a mutant lacking *GRF-INTERACTING FACTOR 1* severely compromises the shoot meristem. We found that miR396 is expressed at low levels throughout the meristem, overlapping with the expression of its target, *GRF2*. In addition, we show that miR396 can regulate cell proliferation and the size of the meristem. *Arabidopsis* plants with an increased activity of the transcription factor TCP4, which reduces cell proliferation in leaves, have higher miR396 and lower *GRF* levels. These results implicate miR396 as a significant module in the regulation of cell proliferation in plants.

Key words: microRNAs, miR396, GRFs, *Arabidopsis*, Cell proliferation, Leaf, TCP4

INTRODUCTION

In contrast to animals, plants continue to produce new organs throughout their life cycle. The above-ground organs are derived from the shoot apical meristem (SAM), which includes a pool of stem cells residing at the growing tip of the plant. Proliferating SAM cells produce an excess of daughter cells that are either incorporated into the developing leaf primordia at the SAM periphery or become part of the shoot. The core machinery controlling the progression of the cell cycle in plants, as well as in other eukaryotes, relies on the activity of cyclin-dependent kinases (Inze and De Veylder, 2006). Many aspects of cell cycle regulation are highly conserved among eukaryotes. It is, however, the integration of the basic cell cycle mechanisms with the developmental program that generates the enormous phenotypic variation among multicellular organisms, a process that is much less understood (Inze and De Veylder, 2006).

In contrast to the indeterminate SAM in *Arabidopsis thaliana*, leaves are determinate organs that have a defined morphology. Leaf development involves the concerted action of various hormone signalling pathways and transcription factor networks. Some of the major transcriptional regulators involved in the control of cell proliferation in leaves include *AINTEGUMENTA* (Mizukami and Fischer, 2000), *PEAPOD* (White, 2006), *JAGGED* (Dinneny et al.,

2004; Ohno et al., 2004), *BLADE ON PETIOLE* (Ha et al., 2003), *TCPs* (Nath et al., 2003) and *GROWTH-REGULATING FACTORS* (*GRFs*) (Kim et al., 2003).

To obtain their characteristic final size and shape, growth of the developing leaf needs to be tightly coordinated first through cell proliferation and then by cell expansion (Piazza et al., 2005; Tsukaya, 2006). Initially, cell proliferation is observed throughout the developing leaf (Donnelly et al., 1999). Then, the cell cycle stops at the tip of the leaf and a mitotic arrest front moves towards the base of the organ (Donnelly et al., 1999). Once cells cease to divide, they begin to enlarge and cell growth becomes the driving force regulating organ size (Piazza et al., 2005; Tsukaya, 2006).

Currently, little is known about the molecular mechanisms that coordinate cell proliferation throughout a developing leaf. A known regulator is the *TCP* gene *CINCINNATA* (*CIN*), which controls the progression of the mitotic arrest front in snapdragon (Nath et al., 2003). Mutations such as *cin* (Nath et al., 2003) and triple knock-outs of its *Arabidopsis* homologues *tcp2/4/10* (Schommer et al., 2008) cause changes in leaf morphogenesis and uneven organ curvature due to excess cell proliferation at the leaf margins. Interestingly, five *Arabidopsis* *TCPs* (*TCP2*, 3, 4, 10 and 24), as well as *CIN*, have a target site for microRNA (miRNA) miR319 (Palatnik et al., 2003). Overexpression of miR319 causes the degradation of these *TCPs* and the generation of crinkled leaves similar to those observed in *tcp* loss-of-function mutants (Palatnik et al., 2003). Mutations in the target site of the *TCPs* that diminish the interaction with the miRNA affect leaf morphology in *Arabidopsis* (Palatnik et al., 2003; Palatnik et al., 2007) and leaf complexity in tomato (Ori et al., 2007), and are lethal in extreme cases (Palatnik et al., 2003).

The *GRF* family of transcription factors comprises nine members in *Arabidopsis* (Kim et al., 2003). Seven of them have a target site for miR396 (Jones-Rhoades and Bartel, 2004). Mutations in

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different *GRFs* or overexpression of miR396 have been shown to reduce cell number in *Arabidopsis* leaves (Horiguchi et al., 2005; Kim et al., 2003; Kim and Kende, 2004; Liu et al., 2009). The *GRFs* work together with *GRF-INTERACTING FACTORS* (*GIFs*), a small gene family encoding proteins with homology to the human SYT transcriptional co-activator (Horiguchi et al., 2005; Kim and Kende, 2004). Inactivation of *GIF1* (Kim and Kende, 2004), also known as *ANGUSTIFOLIA 3* (*AN3*) (Horiguchi et al., 2005), produces narrower leaves as a result of a reduction in cell proliferation.

Here, we describe the role of miR396 in the regulation of the *GRFs*. We show that miR396 accumulates during leaf development and limits the domain of the proliferative tissue. The balance between miR396 and the *GRFs* quantitatively regulates cell number in leaves. Furthermore, we show that miR319-regulated *TCP4* induces miR396 and represses *GRF* activity. We propose that miR396 can operate as a link between the developmental programs of the cell and the more basic cell cycle mechanisms to fine-tune cell proliferation.

MATERIALS AND METHODS

Plant material

Arabidopsis ecotype Col-0 was used for all experiments. Plants were grown in long photoperiods (16 hour light/8 hour dark) or in short photoperiods (8 hour light/16 hour dark) at 23°C.

Transgenes

See Table S1 in the supplementary material for a list of binary plasmids used in this study. The miRNA target motif in *GRF2* was altered, introducing synonymous mutations in a cloned *GRF2* wild-type genomic fragment, using the QuikChange site-directed mutagenesis kit (Stratagene).

Expression analysis

First, 0.5–1.0 µg of total RNA was treated with RQ1 RNase-free DNase (Promega). Then, first-strand cDNA synthesis was carried out using SuperScript III reverse transcriptase (Invitrogen). PCR reactions were performed in a Mastercycler ep *realplex* thermal cycler (Eppendorf) using SYBR Green I (Roche) to monitor double-stranded (ds)DNA synthesis. Quantitative (q)PCR of each gene was carried out for at least three biological replicates, with technical duplicates for each biological replicate. The relative transcript level was determined for each sample, normalized using *PROTEIN PHOSPHATASE 2A* cDNA level (Czechowski et al., 2005). Primer sequences are given in Table S2 in the supplementary material.

Small RNA analysis

RNA was extracted using TRIzol reagent (Invitrogen). Total RNA was resolved on 17% polyacrylamide gels under denaturing conditions (7 M urea). Blots were hybridized using either radioactively labelled or digoxigenin end-labelled locked nucleic acid (LNA) oligonucleotide probes designed against miR396 (Exiqon, Denmark).

Alternatively, miR396 levels were determined by stem-loop RT-qPCR, as described previously (Chen et al., 2005). The sequences of the oligonucleotides used were: retrotranscription stem-loop oligo, 5'-GTCTCCTCTGGTGCAGGGTCCGAGGTATTTCGACACAGAGGAG-ACMAGTTC3'; PCR forward primer, 5'-GGCGGTTCCACAGCTTT-CTT3'; and PCR reverse primer, 5'-TGGTGCAGGGTCCGAGGTATT3'.

Microarray analyses

Total RNA was extracted from the aerial part of seedlings grown on plates for 10 days using the RNeasy plant mini kit (QIAGEN). Microarray analyses using the Affymetrix ATH1 platform were performed on two biological replicates as described (Schmid et al., 2005). Differentially expressed genes were identified using per-gene variance, calculated using logit-T (Lemon et al., 2003). The corresponding fold change of the transcripts was obtained by expression estimates using gcRMA (www.bioconductor.org), a modification of the robust multi-array analysis (RMA) algorithm (Irizarry et al., 2003). The expression of gene groups was assessed by gene set enrichment analysis using GSEA-P 2.0 (Subramanian et al., 2007; Subramanian et al., 2005).

Microscopic observations

Tissue was fixed in FAA and embedded in paraffin. Sections 10 µm thick were stained with Toluidine Blue.

To obtain paradermal views of palisade cells, leaves were fixed with FAA and cleared with chloral hydrate solution as described (Horiguchi et al., 2005). Palisade leaf cells were observed using differential interference contrast (DIC) microscopy.

In situ hybridization

DIG-labelled sense and antisense probes were synthesized by transcription with T7 or SP6 RNA polymerase with the DIG RNA labelling kit (SP6/T7) (Roche) using cloned cDNAs of *GRF2* and *HISTONE H4* as templates. For the miR396 probe, LNA oligonucleotides (Exiqon) were end labelled with the DIG oligonucleotide 3'-end labelling kit (Roche). Shoot apices from 15-day-old plants grown in short photoperiods were dissected and fixed in FAA. Paraffin-embedded material was sectioned to 8 µm thickness. Hybridization and detection were performed as previously described (Palatnik et al., 2003).

GUS assays

To visualize the activity of the reporters, transgenic plants were subjected to GUS staining, according to Donnelly et al. (Donnelly et al., 1999). Stained tissue was paraffin embedded, sectioned and mounted in Canada balsam.

Accession numbers

A list of relevant AGI locus identifiers is provided in Table S2 in the supplementary material. The accession number for the microarray experiments is GSE11250.

RESULTS

miR396 accumulates during leaf development

Seven of the nine *Arabidopsis* *GRFs*, *GRF1-4* and *GRF7-9*, have a sequence that is partially complementary to that of miR396 (Fig. 1A). The miR396 target sequence is located at the end of a region that encodes a conserved WRC domain (Fig. 1A). The *GRF* alignments to miR396 share a bulge at position 7 (counting from the 5' end of the miRNA), whose identity varies among the target genes (Fig. 1A).

To characterize the functional role of miR396, we followed the expression pattern of the miRNA and its targets during leaf development. We collected the fifth rosette leaf at three-day intervals, starting from the day that it first became visible (~1 mm) to the naked eye, which was 16 days after sowing (DAS). We performed small RNA blots on these samples and found that miR396 was expressed at low levels in young leaves and that it steadily accumulated during the development of the organ (Fig. 1B).

To accurately quantify the expression levels of the miRNA, we turned to an RT-qPCR approach (Chen et al., 2005). We found that miR396 levels increased up to 30-fold during leaf development (Fig. 1C). We also measured the level of the *MIR396a* precursor and found that it was also upregulated during organogenesis, in good correlation with the level of the mature miRNA (Fig. 1C).

Next, we determined the level of the miR396-regulated *GRFs* by RT-qPCR. We observed that these transcription factors were expressed during the early stages of leaf development (Fig. 1D). Then, we observed a decrease in the level of miR396-regulated *GRFs* during leaf development that was complementary to the increase in the miRNA level (Fig. 1B-D).

We then analyzed the expression of *GRF5* and *GRF6*, which are not regulated by miR396. We found that these *GRFs* had a similar expression pattern to the miR396-regulated transcription factors during organ growth (Fig. 1E). To correlate the expression pattern of the *GRFs* with the developmental stage of the leaf, we determined the levels of *CYCLINB1;1* (*CYCB1;1*) and *KNOLLE*, which are known to be expressed during mitosis (Lukowitz et al., 1996;

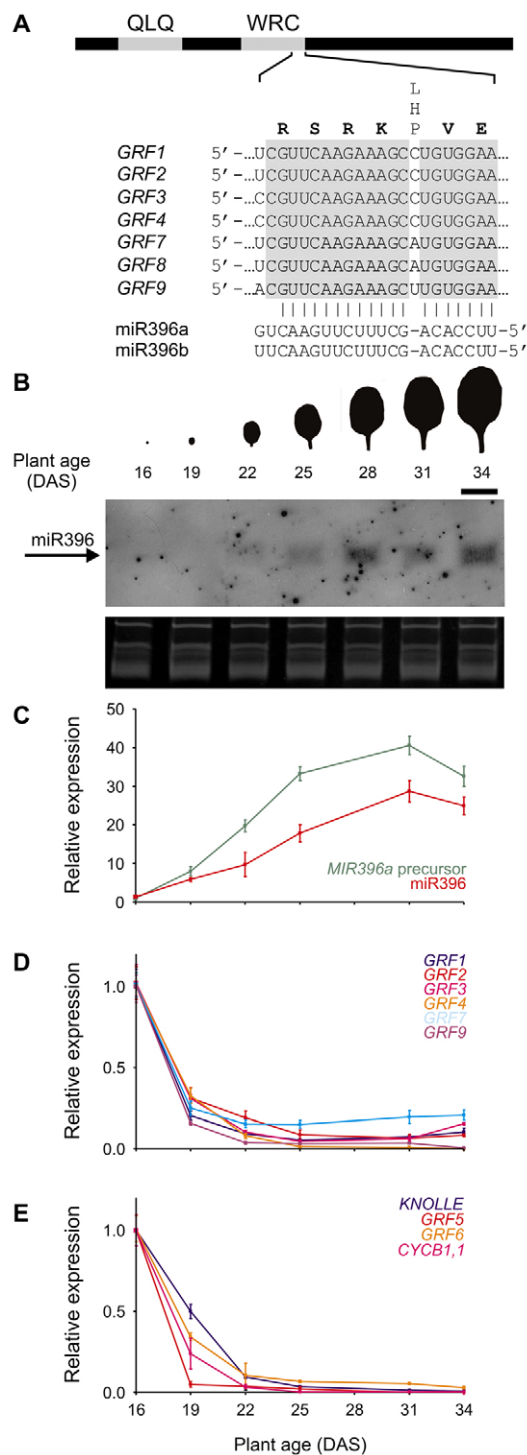


Fig. 1. Accumulation of miR396 during leaf development.

(A) Scheme representing the *GRF* genes. The interaction of the seven miRNA-regulated *GRFs* from *Arabidopsis* with miR396 is shown. QLQ and WRC indicate the conserved domains that define the *GRF* family (Kim et al., 2003). (B) Pattern of miR396 accumulation during development of leaf 5. Samples were collected every 3 days starting 16 DAS. miR396 accumulation was analyzed by small RNA blots. Scale bar: 1 cm. (C) Accumulation of *MIR396a* precursor and mature miR396 during development of leaf 5. Both precursor and mature miRNA were detected by RT-qPCR. The expression levels were normalized to the earliest time point. The data shown are mean \pm s.e.m. of three biological replicates. (D) Expression of miR396-regulated *GRFs* during leaf development. The *GRFs* were detected by RT-qPCR. Determination procedures were carried out as in C. (E) Expression of *GRF5-6*, *GIF1*, *KNOLLE* and *CYCB1;1* during leaf development. Determination procedures were carried out as in C.

downstream of the miR396 target site, in the *GRF2* genomic region including 2 kb upstream of the 5'-UTR (Fig. 2A). This wild-type reporter (*GRF2:wtGRF2-GUS*) should monitor *GRF2* transcriptional and post-transcriptional regulation.

During organ growth, *GRF2:wtGRF2-GUS* was strongly expressed in young leaves (Fig. 2B). At later stages of development, reporter activity was detected mainly at the base of the leaf, with a gradient that tapered off towards the distal part of the leaf (Fig. 2B). Finally, we did not observe activity of the wild-type *GRF2* reporter in fully expanded leaves. This dynamic pattern of expression of the reporter was repeated with the generation of each new leaf (Fig. 2B).

To reveal the activity of miR396 in leaves and its effect on the expression pattern of *GRF2*, we prepared a second reporter, with synonymous mutations in the miR396 target site that disrupt the interaction with the miRNA (Fig. 2A). Compared to the wild-type reporter, the expression domain of *GRF2:rGRF2-GUS* was extended in the leaves of 14 out of 20 T1 lines (Fig. 2C), indicating that the *GRF2* promoter is apparently uniformly active throughout young leaves. As the reporters differ only in their sensitivity to the miRNA, the concomitant difference in their expression pattern should reflect the activity of miR396 *in vivo*. Therefore, these results indicated that miR396 accumulates during leaf development (Fig. 2B,C); in good agreement with our previous results (Fig. 1).

At a given developmental time, each individual rosette leaf showed a particular expression level of the wild-type *GRF2* reporter (Fig. 2B). We determined the levels of miR396 in each leaf and found that the miRNA was expressed at the highest level in the oldest leaf, in good correlation with the activity of the reporters (Fig. 2D). RT-qPCR experiments with these samples showed that miR396 levels vary up to tenfold between the apices (containing the smallest leaves) and fully expanded leaves (Fig. 2F). The levels of the *MIR396a* and *MIR396b* precursors were also increased tenfold, suggesting that both miRNA genes have a redundant pattern of expression (Fig. 2F). The *GRFs*, together with cell proliferation markers such as *CYCB1;1* and *KNOLLE*, were expressed in young proliferative tissues, whereas their expression decreased in older tissues, contrasting with the expression pattern of miR396 (Fig. 2G,H).

During the initial stages of leaf development, the wild-type *GRF2* reporter displayed a high and homogeneous level of expression, whereas in mature leaves the reporter was turned off. Interestingly,

Menges et al., 2005). We observed that the expression of these cell cycle markers decreased in a similar way to the *GRFs* during early leaf development (Fig. 1E).

Dynamic pattern of miR396 expression

Our previous results showed that miR396 and the *GRFs* have contrasting expression patterns during leaf development. To study the expression of miR396 in more detail and uncover its function in the regulation of the *GRFs*, we prepared two reporters. First, we inserted the β -glucuronidase (*GUS*) gene in frame after exon 4,

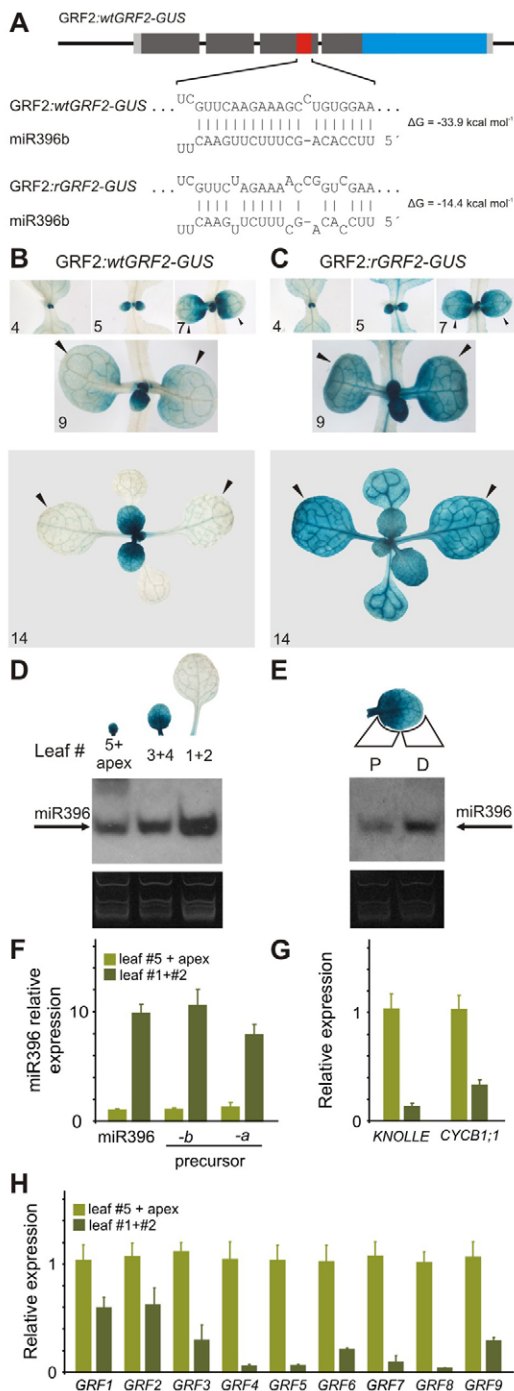


Fig. 2. Dynamic pattern of miR396 expression. (A) Scheme representing two reporters with different sensitivity to miR396: *GRF2:wtGRF2-GUS*, which is sensitive to the miRNA, and *GRF2:rGRF2-GUS*, which is insensitive. (B) GUS staining of *GRF2:wtGRF2-GUS* plants. Arrowheads point to leaves 1 and 2. Numerals indicate plant age expressed as DAS. (C) GUS staining of *GRF2:rGRF2-GUS* plants. Arrowheads point to leaves 1 and 2. Numerals indicate plant age expressed as DAS. (D) Accumulation of mature miR396 in different leaves of a rosette. The *GRF2:wtGRF2-GUS* pattern of the same leaves is indicated at the top of each lane. (E) Expression of miR396 in the proximal (P) and distal (D) parts of leaf 3 (taken from same plants as for panel D). (F-H) Quantification of miR396, cell proliferation markers and *GRFs* in proliferative (leaf 5 plus apex) and non-proliferative (leaves 1 and 2) organs by RT-qPCR. The expression levels were normalized to the sample: leaf 5 plus apex. The data shown are mean \pm s.e.m. of three biological replicates. (F) Accumulation of *MIR396a* and *MIR396b* precursors and mature miR396. (G) *KNOLLE* and *CYC1;1*. (H) *GRF1-9*.

the proximo-distal axis of developing leaves, with higher expression levels in the distal part. This suggests that miR396 could also be a marker along the longitudinal axis of a single organ.

Effects of altered miR396 levels on *GRF* expression and leaf development

To gain insights into the biological role of miR396, we prepared transgenic plants with increased levels of the miRNA by expressing *MIR396b* under the control of the viral 35S promoter. Most of the transgenic plants (63 out of 81 T1 plants) had smaller rosettes (Fig. 3A,B). This is in good agreement with previous observations showing that *GRF* mutants (Horiguchi et al., 2005; Kim et al., 2003) or high miR396 levels reduce leaf size (Liu et al., 2009).

We then measured the levels of the *GRFs* in 35S:*miR396b* stable transgenics by RT-qPCR. We observed that the high levels of miR396 caused a reduction in the expression of the miRNA-regulated transcription factors; however, the magnitude of the decrease in their mRNA levels varied among the different *GRFs* (Fig. 3C). Similar results have been obtained from the overexpression of other miRNAs (Schwab et al., 2005).

GRF5 and *GRF6*, which do not have a miR396 target site, responded in different ways to miR396 overexpression. Whereas *GRF5* was largely unaffected, *GRF6* expression was decreased (Fig. 3C). These results suggested a feed-forward effect of the miR396-regulated *GRFs* on the levels of *GRF6* in 35S:*miR396b* plants or that the expression of *GRF6* is affected by the developmental changes caused by miR396.

Previous results have indicated that mutations in *GRF5* produced moderately smaller leaves with fewer cells (Horiguchi et al., 2005). Primary transgenics harbouring an artificial miRNA against *GRF5* and *GRF6* also revealed a moderate effect on leaf development (V. Crosa and J.F.P., unpublished). Our results indicated that the miR396-regulated *GRFs* and *GRF5-6* were expressed in a similar way during leaf development (Fig. 1), which might suggest that they have redundant functions. Therefore, we overexpressed miR396 in the context of a *grf5* insertional mutant (for a list of mutants used in this work, see Table S2 in the supplementary material). Transgenic plants with this large decrease in *GRF* activity were severely affected, and their leaves were smaller and narrower, reflecting synergistic effects between the 35S:*miR396b* transgene and *grf5* (Fig. 3B).

at intermediate developmental stages, the wild-type *GRF2* reporter showed a proximo-distal gradient of expression; this is in contrast to the miRNA-resistant *GRF2* reporter, which was expressed throughout the leaf (Fig. 2B,C). To test whether miR396 was differentially expressed at the tip and the base of the leaf, we dissected a developing leaf and determined the level of miR396 in the proximal and distal parts. Small RNA blots confirmed that miR396 accumulated to higher levels in the distal part of the leaf (Fig. 2E).

Taken together, these results indicate a direct relationship between miR396 levels and the developmental age of the organ, with high levels in mature organs. Furthermore, miR396 activity varied along

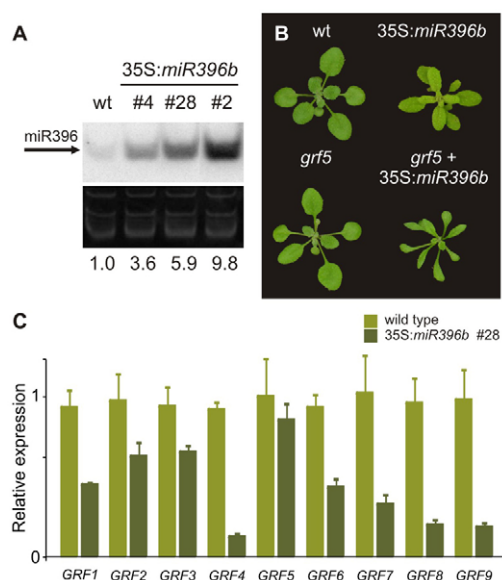


Fig. 3. Effects of miR396 on *GRF* expression and leaf development. (A) Expression of miR396 in 10-day-old rosettes of wild-type plants and miR396b overexpressers. Numerals indicate the miR396 content of transgenic plants relative to that of wild-type plants. (B) Effect of miR396 overexpression on wild-type plants and *grf5* mutants. (C) Expression levels of *GRFs* in 10-day-old rosettes of wild-type and 35S:miR396b #28 plants. The *GRF* levels were determined by RT-qPCR and normalized to wild-type plants. The data shown are mean \pm s.e.m. of five biological replicates.

Genome-wide response to miR396

To identify molecular targets of the miR396 network, we compared global expression profiles in the aerial parts of 10-day-old wild-type and 35S:miR396b #28 seedlings using Affymetrix microarrays. We chose a relatively early stage of a moderate miR396 overexpresser to minimize the consequences of the different final anatomy caused by high miRNA levels. As expected, the expression levels of the miR396-regulated *GRFs* were lower in 35S:miR396b seedlings (see Table S3 in the supplementary material). Using a per-gene variance of $P < 0.05$ calculated using the logit-T algorithm (Lemon et al., 2003), we found only 12 genes that were downregulated in 35S:miR396b plants (see Table S4 in the supplementary material).

Interestingly, *KNOLLE* was one of the genes downregulated in 35S:miR396b. *KNOLLE* expression is coordinated with 79 other *Arabidopsis* genes present on the ATH1 array that have peaks of expression when cells enter mitosis (Menges et al., 2005), probably through mitosis-specific activator elements in their promoters (Haga et al., 2007; Menges et al., 2005). Therefore, we further analyzed the expression of all mitosis-specific genes using Gene Set Enrichment Analysis, a test designed to detect changes in the expression of coordinated groups of genes (Subramanian et al., 2005). We found that the mitosis-specific genes were significantly downregulated as a group in 35S:miR396b compared to wild-type plants (nominal $P < 0.0001$, false discovery rate < 0.0001 , see Tables S5 and S6 in the supplementary material).

To validate the microarray results experimentally, we analyzed a cyclin reporter (Donnelly et al., 1999). The expression patterns of the *CYCB1;1* and the wild-type *GRF2-GUS* reporters were very similar (Fig. 4A,B). In good agreement with this, a closer look at the

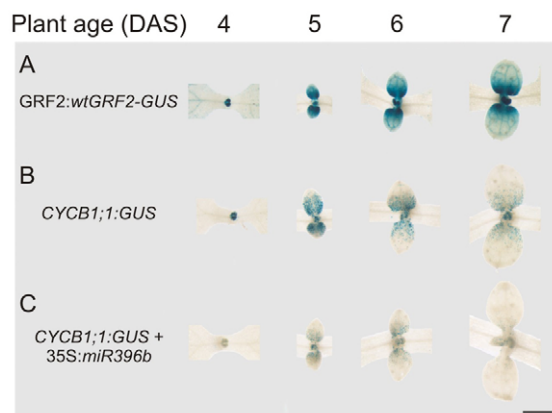


Fig. 4. Control of cell proliferation by miR396. (A) *GRF2:wtGRF2-GUS* expression 4-7 DAS. (B) *CYCB1;1:GUS* reporter expression in plants the same age as in A. (C) *CYCB1;1:GUS* reporter expression in 35S:miR396b #28 transgenic plants. Scale bar: 1 mm.

gradient of wild-type *GRF2-GUS* revealed that the reporter activity started to fade away before cell expansion begins (see Fig. S1 in the supplementary material).

We then analyzed *CYCB1;1* in plants overexpressing miR396 and observed that high levels of the miRNA greatly reduced the number of *CYCB1;1* positive cells in developing leaves (Fig. 4C), confirming the results of the microarray analysis. Furthermore, cycling cells were closer to the base of developing leaves in 35S:miR396b than in the wild type, suggesting that the mitotic arrest front occurs earlier in transgenic plants with high miRNA levels (Fig. 4B,C).

The balance between miR396 and the *GRFs* quantitatively regulates leaf cell number

To determine the requirement for miR396 to restrict *GRF* activity during leaf development, we introduced two *GRF2* genomic fragments into plants, one with the wild-type *GRF2* coding sequence and one in which the miRNA-targeting motif was altered through synonymous mutations that should prevent miR396 targeting (*rGRF2*) (Fig. 5A). We analyzed several independent insertion lines for each construct at seedling stage, when the levels of miR396 are low, and found that additional copies of wild-type or miRNA-resistant *GRF2* transgenes raised the *GRF2* mRNA level several fold (Fig. 5A). However, the *rGRF2* version led to higher levels of mRNA accumulation compared to the wild-type transgene, up to 20-fold (Fig. 5A).

The reduction in leaf size caused by miR396 overexpression was suppressed by the introduction of *rGRF2*, demonstrating that leaf development was disturbed in 35S:miR396b plants because of a decrease in *GRF* activity (Fig. 5B-D, see Fig. S2 in the supplementary material). Plants expressing *rGRF2* in a wild-type background had larger leaves with more cells than control plants (Fig. 5E-I). Transgenic lines expressing high levels of wild-type *GRF2* also had a moderate increase in cell number (see Fig. S2 in the supplementary material), which is in good agreement with a quantitative interaction between miR396 and the *GRFs*. However, we noticed that cells in *rGRF2* leaves could also be reduced in their size (Fig. 5H), an effect that we have not observed in plants expressing the wild-type version of the transcription factor.

35S:miR396b plants with the highest levels of miR396 (Fig. 3A) had fewer palisade cells in the subepidermal layer (Fig. 5E-I). In these cases, the final number of cells in leaf 1 was estimated to be

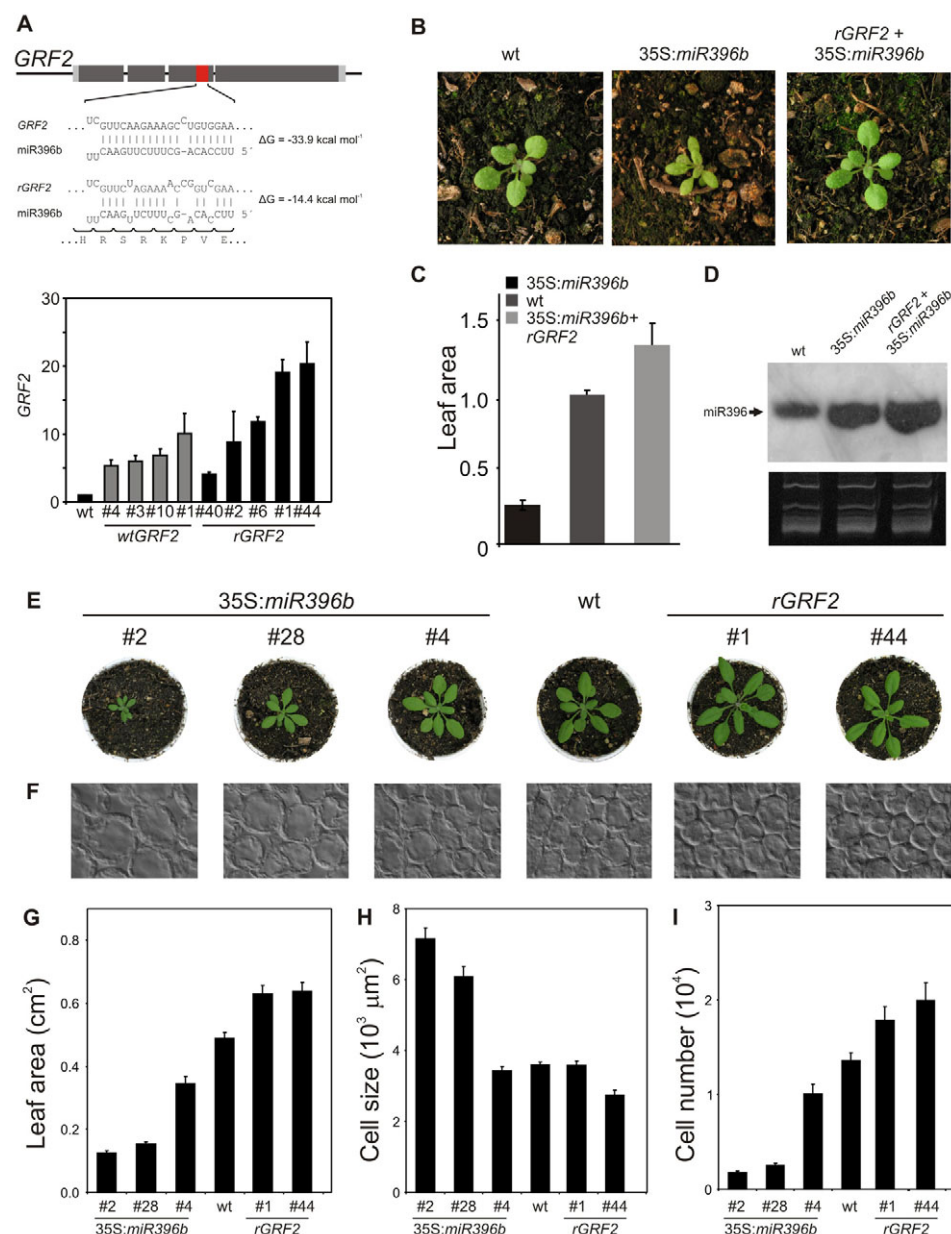


Fig. 5. Regulation of leaf development by the balance between miR396 and the GRFs. (A) Diagram of *rGRF2* indicating the synonymous mutations that alter the interaction with miR396. The graph below shows expression levels of *GRF2* in seedlings with extra copies of a wild-type (*GRF2*) or miRNA-resistant (*rGRF2*) form of the transcription factor. The data shown are mean \pm s.e.m. of three biological replicates. (B-D) Complementation of the miR396 overexpression phenotype by *rGRF2*. (B) Rosette phenotype of wild-type, *35S:miR396b* and *rGRF2* plants overexpressing miR396. (C) Leaf area determined for fully expanded first leaves of wild-type, *35S:miR396b* and *rGRF2+35S:miR396b* plants. Values expressed relative to wild-type plants. (D) miR396 expression in wild-type, *35S:miR396b* and *rGRF2* plants overexpressing miR396. (E) Rosette phenotype of 18-day-old plants with different *GRF* levels. *35S:miR396b* plants have reduced *GRF* levels, whereas *rGRF2* plants have increased *GRF* levels. (F) Paradermal view of palisade cells in the subepidermal layer of leaf 1 of 20-day-old plants. (G-I) Leaf area (G), cell size (H) and palisade cell number (I) per leaf in wild-type, *35S:miR396b* and *rGRF2* transgenic plants. The data shown are the mean \pm s.e.m. of eight leaves or 50 cells.

less than 13% that of the wild type (Fig. 5I). Concomitant with this reduction in leaf cell number was an increase in cell size: cells from *35S:miR396b* plants were twice as large as wild-type cells (Fig. 5H). Determination of leaf area, cell size and palisade cell number in the transgenic plants indicated a quantitative effect of miR396 levels on the leaf parameters (Fig. 5E-I).

Cell size, however, was only altered in plants with at least a fivefold increase in miR396, which partially compensated for the reduction in cell number (Fig. 5). Compensatory changes during leaf development are well known and have been observed in *an3* mutants or strong overexpressors of the cyclin-dependent kinase inhibitor *KIP-RELATED PROTEIN2* (De Veylder et al., 2001; Ferjani et al., 2007; Fujikura et al., 2009; Horiguchi et al., 2006; Tsukaya, 2005). Such changes have also been shown to be activated once the cell number deviates beyond a certain threshold. Taken together, our results suggest that the balance between miR396 and the *GRFs* quantitatively regulates cell proliferation in leaves.

Regulation of cell proliferation by miR396 in the SAM

In addition to their post-transcriptional regulation by miR396, the *GRFs* have been shown to interact with GIF proteins, which have been proposed to act as transcriptional co-activators (Horiguchi et al., 2005; Kim and Kende, 2004). The *an3-1* mutant, which has a mutation in *GIF1*, has smaller leaves and petals (Horiguchi et al., 2005; Kim and Kende, 2004). To further decrease the *GRF* activity, we overexpressed miR396 in *an3-1* and observed that half of the *35S:miR396b an3-1* plants (48 out of 90 independent T1s) lacked a functional SAM (Fig. 6A). Sections revealed the absence of a meristematic dome and developing leaf primordia (Fig. 6B). These results indicated synergistic effects between miR396 overexpression, which post-transcriptionally regulates the *GRFs*, and mutations in the co-activator *GIF1*, and also suggested functions of miR396 in SAM development and maintenance.

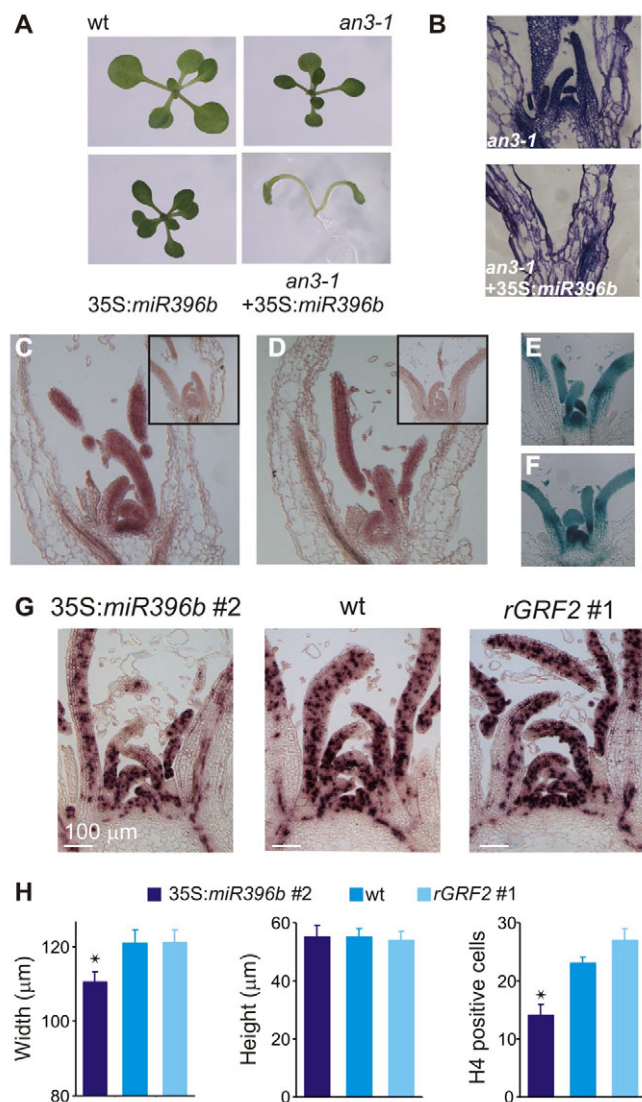


Fig. 6. Control of meristem function by miR396. (A) Effect of miR396 overexpression on *an3-1* mutants. (B) Medial-longitudinal sections of 15-day-old seedlings stained with toluidine blue. Note the lack of a meristem dome in 35S:miR396b *an3-1* plants. (C) In situ hybridization of vegetative shoot apices with an miR396 probe in the wild type. The inset shows an in situ hybridization to a section of *hyl1-1* mutant, which has reduced miRNA levels. (D) In situ hybridization of vegetative shoot apices with an antisense probe against *GRF2* mRNA. An in situ hybridization with a sense probe is shown in the inset. (E-F) Expression pattern in sections of shoot apices of *GRF2:wtGRF2-GUS* (E) and *GRF2:rGRF2-GUS* (F) reporters. (G) *HISTONE H4* expression in shoot apices of 30-day-old plants grown in short photoperiods, detected by in situ hybridization. (H) Morphology and cell proliferation of the SAM. Width and height were estimated from medial-longitudinal sections of the SAMs of 30-day-old plants grown in short photoperiods. *HISTONE H4* positive cells were counted from sections such as those of panel G. Numbers are mean \pm s.e.m. ($n=9$). Asterisks indicate significant differences between transgenic and wild-type plants, as determined by ANOVA ($P<0.05$).

These results prompted us to analyze the role of miR396 and the *GRFs* in meristem function. First, we studied the expression of miR396 by in situ hybridization on sections of 15-day-old plants grown in short photoperiods. We detected miR396

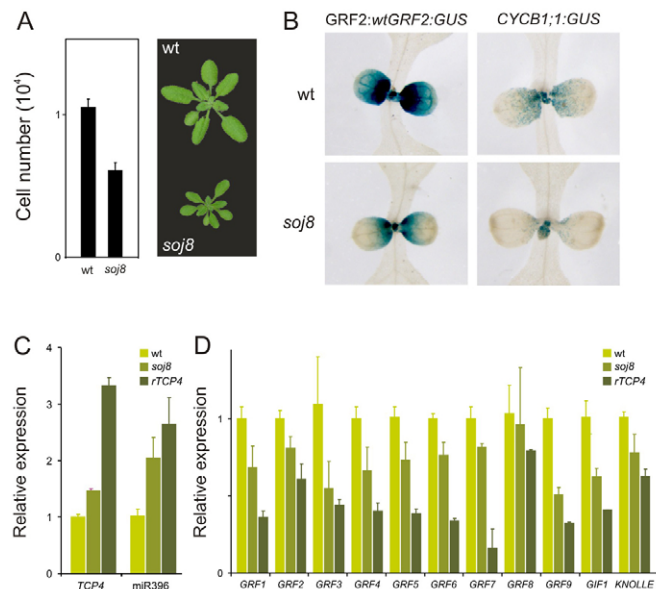


Fig. 7. Regulation of miR396 and the *GRFs* by *TCP4*. (A) Estimated palisade cell number per leaf in wild-type and *soj8* plants. The data shown are the mean \pm s.e.m. of eight leaves or 50 cells. (B) *GRF2:wtGRF2-GUS* and *CYCB1:1-GUS* expression in wild-type and *soj8* plants. (C) Accumulation of *TCP4* and miR396 in moderate *rTCP4* transgenic plants and *soj8* mutants. Determinations were carried out by RT-qPCR and normalized to wild-type plants. RNA was prepared from apices of 30-day-old plants grown in short photoperiods, including developing leaves smaller than 3 mm. The data shown are mean \pm s.e.m. of three biological replicates. (D) Measurements of *GRF1-9*, *GIF1* and *KNOLLE* in *soj8* and *rTCP4* plants. Samples were analyzed as in C.

throughout the SAM and leaf primordia (Fig. 6C). We also observed the expression of *GRF2* in the meristem and young developing leaves (Fig. 6D). In good agreement with this, the GUS staining pattern in sections of wild-type *GRF2-GUS* was similar to the expression of *GRF2* mRNA (Fig. 6D,E). At this stage, we did not see any qualitative difference between the expression patterns of the wild-type and miR396-resistant *GRF2* reporters (Fig. 6E,F). Taken together, these results indicated that there is weak expression of miR396 at the SAM and leaf primordia, which overlaps with *GRF2* expression.

Next, we investigated whether miR396 overexpression, which we have shown to affect leaf growth by reducing cell proliferation, also compromised the function of the SAM. We performed in situ hybridizations with *HISTONE H4*, which is expressed during the G1-S phase of the cell cycle in coordination with DNA synthesis (Gaudin et al., 2000; Krizek, 1999). We found that 35S:miR396 plants had fewer proliferating cells in the SAM, paralleled with a reduction in SAM size (Fig. 6G,H). The effects on SAM size indicated that the miR396-targeted *GRFs* can affect meristem function.

The effect of miR396 on the proliferation of meristematic cells is consistent with the lack of a functional meristem in 35S:miR396b *an3-1* plants. Previous studies have shown that some of the progeny of *grf* multiple mutants have cotyledon fusions (Kim et al., 2003; Kim and Lee, 2006), which often appear to be related to defects in SAM function. Taken together, these data implicate the network formed by miR396, the *GRFs* and their interacting factors as a relevant regulator of meristem function, at least partially through the control of cell proliferation.

Regulation of the miR396 network by *TCP4*

The dynamic expression pattern of miR396, which generates a distal-proximal gradient of miRNA activity, and its role in the repression of cell proliferation suggested that miR396 could be part of the mitotic arrest front during leaf development. Interestingly, *TCP* transcription factors with a target binding site for miR319 have already been implicated in the regulation of the arrest front (Nath et al., 2003). Therefore, we decided to test the interaction between these two miRNA networks.

We analyzed two genotypes: *soj8*, an EMS-generated line that contains a point mutation in the miRNA target site of *TCP4* that diminishes the interaction with miR319 (Palatnik et al., 2007); and transgenic plants moderately overexpressing *TCP4::rTCP4-GFP* (see Fig. S3 in the supplementary material), as higher levels of *TCP4* are lethal (Palatnik et al., 2003; Schommer et al., 2008). We observed that the mild increase in *TCP4* levels in *soj8* (Fig. 7C) caused a reduction in cell number, with a concomitant reduction in leaf size (Fig. 7A). Analysis of the *CYCBI;1* reporter in *soj8* confirmed a reduction in the number of cycling cells (Fig. 7B).

We then measured the levels of miR396 and found that they were increased two- to three-fold in the apices of *soj8* and *TCP::rTCP4-GFP* (*rTCP4*) with respect to wild-type plants (Fig. 7C). The increase in miR396 levels was even detected in *soj8*, which has a mild phenotype and a marginal increase in *TCP4* levels of nearly 50% (Fig. 7C). We crossed *soj8* to the *GRF2::wtGRF2-GUS* plants and observed that the reporter was turned off sooner in the mutant than in wild-type plants (Fig. 7B).

Next, we determined the levels of the miRNA-regulated *GRFs* in *soj8* and weak *rTCP4* transgenic plants, and found that there was a reduction in the levels of these transcription factors (Fig. 7D). Interestingly, we observed that the levels of *GRF5* and *GRF6*, which are not regulated by miR396, as well as of *GIF1*, were decreased in *soj8* and *rTCP4* (Fig. 7D), indicating that *TCP4* also regulates GRF activity independently of miR396.

The finding that *TCP4* can inhibit the expression of *GIF1* as well as the *GRFs* agreed with the strong effects of the miR319-resistant *TCP4* on leaf morphology. We observed a similar phenotype in both *rTCP4* and 35S::miR396 *an3-1* transgenic plants that were expressing the transgenes at moderate levels (see Fig. S4 in the supplementary material). It has been suggested that the repression of organ boundary genes by the *TCPs* underlies the lethality of the miR319-resistant *TCPs* (Koyama et al., 2007). Our results suggest that a major inhibition of the cell cycle machinery could also be involved in the harmful effects caused by *rTCP4*.

DISCUSSION

miR396 as a marker of leaf development

We followed the expression pattern of miR396 directly using small RNA blots and in situ hybridization in apices, and indirectly through the differential expression of wild-type and miRNA-resistant *GRF2-GUS* reporters. miR396 was expressed at low levels in the meristem and leaf primordia, and then it steadily accumulated with the development of the leaf. In contrast, the *GRFs*, which are highly expressed in the SAM and young leaves, decreased during leaf development, in concert with the retreat of cell proliferation.

Temporal antagonistic patterns of expression have been observed for miR156 and miR172 and their targets, the SPL and AP2-like transcription factors, respectively (Chuck et al., 2007; Wu and Poethig, 2006). The heterochronic miR156 and miR172 networks correspondingly regulate juvenile to adult, and vegetative to reproductive phase transitions, which require decisions implicating the whole plant (Aukerman and Sakai, 2003; Chen, 2004; Chuck et

al., 2007; Schmid et al., 2005; Wu and Poethig, 2006). Our observations on miR396 indicated that this miRNA is also involved in the coordination of developmental events in plants; however, its role would be restricted to individual organs.

The *Arabidopsis* developmental program directs a basiplastic pattern, whereby leaf maturation begins at the tip and then proceeds towards the base of the organ (Donnelly et al., 1999). Cell division occurs first throughout the primordia and then a mitotic arrest front moves from the tip to the base of the leaf, so that cells in the distal part of the leaf stop cycling and begin to expand, while cells at the base continue to proliferate (Donnelly et al., 1999). Our results showed that the distal part of the leaf accumulates more miR396 and a gradient of miRNA activity proceeds towards the base of the organ. This result was supported by small RNA blots and the observed retreat of the wild-type *GRF2-GUS* reporter, which then matched the pattern of a *CYCBI;1* reporter. These observations prompt us to implicate the repression of *GRF* expression by miR396 as a component of the mitotic arrest front.

Regulation of the *GRFs* by miR396

We observed similar spatial patterns of expression for *GRF2* mRNA and miR396 in the meristem and leaf primordia, indicating that there is co-expression of the miRNA and its target at this early stage. The situation was different, however, at later stages of leaf development. The wild-type *GRF2-GUS* reporter was active only in the proximal part of young developing leaves, whereas the *rGRF2-GUS* reporter was expressed throughout the leaf. This qualitative change in the expression of wild-type *GRF2-GUS* was paralleled by a large increase in miR396, whose levels change by up to 10–30-fold in leaves with different developmental ages. Interestingly, the decrease in *GRF* expression occurred before miR396 reached its maximum level, indicating that a partial increase in the miRNA is sufficient to repress the *GRFs* in vivo; however, we cannot rule out the participation of additional factors that act in concert with miR396.

It has been proposed that miRNAs could have both qualitative effects, leading to complete elimination of their targets, and more subtle quantitative effects (Bartel and Chen, 2004). In plants, these quantitative interactions have been proposed for miR169 (Cartolano et al., 2007) and miR156 (Wang et al., 2008), miR319 (Ori et al., 2007) and miR164 (Baker et al., 2005; Nikovics et al., 2006), and their targets. From a mechanistic point of view, it is tempting to speculate that miR396 has dual functions during leaf development: it might quantitatively regulate *GRF* expression in the SAM and leaf primordia, while causing a large qualitative effect contributing to the clearance of GRF activity from older organs. This latter functional role in clearing *GRF* transcripts might explain the continued rise in miR396 levels, even after cell proliferation has ceased. On the other hand, the potential quantitative regulation of GRF activity during early leaf development might play a relevant role in the fine-tuning of cell proliferation, as we have shown that modifications of the balance between miR396 and *GRF2* levels have important consequences for the final number of cells in the organ.

Control of cell proliferation by the miR396 network

miR396 was first identified because of its conservation between *A. thaliana* and rice (Jones-Rhoades and Bartel, 2004). miR396 and *GRFs* with an miR396 target site are present in many plant species (Axtell and Bartel, 2005; Jones-Rhoades and Bartel, 2004), indicating an ancient origin for the miR396-GRF regulatory

network. The function of the GRFs as regulators of cell number in leaves is well established based on the phenotypes of *grf* (Horiguchi et al., 2005; Kim et al., 2003; Kim and Lee, 2006) and *gif* (Horiguchi et al., 2005; Kim and Kende, 2004) mutants, and plants with high miR396 levels (Liu et al., 2009).

Here, we have extended these observations and found that the *GRFs* regulate cell proliferation in the SAM, which at least partially explains the lack of a functional meristem in *an3-1* mutants overexpressing miR396 (this study) and in *grf* multiple knock-outs (Kim et al., 2003; Kim and Lee, 2006). Analysis of the transcriptome of moderate miR396 overexpressers has shown that the downregulation of mitosis-specific genes is one of the main molecular effects of high miR396 levels. However, the *GRFs* themselves do not change their expression during the cell cycle (Menges et al., 2005) and future work will be required to identify the mechanisms underlying the activity of the GRFs.

Measurements of the *GRFs* by RT-qPCR indicated that miR396 targets and non-targets are turned off at similar stages of leaf development, and that they act redundantly. Previous studies in which promoters have been fused directly to a GUS reporter have shown that the transcription of the *GRF* genes can occur in different regions of the leaf (Horiguchi et al., 2005). We observed that the post-transcriptional control of *GRF2* by miR396 contributes significantly to its final expression pattern, so it is possible that the miRNA also plays a key role in adjusting the expression of other *GRFs*.

Regulation of leaf development by *TCP4* and miR396

The snapdragon *TCP* gene *CIN* has been shown to be expressed in a dynamic pattern during leaf development and to regulate cyclin expression (Nath et al., 2003). *CIN*-like genes from *Arabidopsis*, which are regulated by miR319, have also been implicated in the coordination of cell proliferation and differentiation in leaves (Efroni et al., 2008; Koyama et al., 2007; Masuda et al., 2008; Palatnik et al., 2003; Schommer et al., 2008). An increase of *TCP4* levels due to mutations that impair the interaction with miR319 produces smaller leaves (Efroni et al., 2008; Palatnik et al., 2003; Schommer et al., 2008).

Here, we observed that plants expressing miR319-resistant forms of *TCP4* induced miR396. As the quantitative balance between miR396 and the *GRFs* regulates cell number in leaves, the increase in miR396 caused by *TCP4* might be responsible for at least part of the reduction in cell number in *soj8* mutants. We observed, however, that the increase in *TCP4* levels also caused a reduction in the *GRFs* that were not regulated by miR396 and *GIF1*, indicating an effect at the transcriptional level. Regulatory circuits in which a transcription factor causes both the transcriptional repression of target genes and the induction of an miRNA that in turn post-transcriptionally inhibits the same group of genes are well described in animals, where they are referred to as coherent feed-forward loops (Hornstein and Shomron, 2006).

miR319 overexpressers (Efroni et al., 2008; Ori et al., 2007; Palatnik et al., 2003) and *tcp* knock-outs (Nath et al., 2003; Schommer et al., 2008) have large changes in leaf morphology, as well as other phenotypic defects, such as a delay in flowering time (Palatnik et al., 2003). This indicates that the *TCPs* have functions that go beyond leaf development. However, it may be possible that the miR319-regulated *TCPs* recruit the miR396 network as part of their biological function. We proposed here that the miR396 network could be a link between different developmental inputs or environmental stimuli and the components of the cell cycle machinery.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.043067/-/DC1>

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