Molecular Mechanism of microRNA396 Mediating Pistil Development in Arabidopsis\(^1\)[W]

Gang Liang, Hua He, Yang Li, Fang Wang, and Diqiu Yu*

Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, Yunnan 650223, China (G.L., H.H., Y.L., F.W., D.Y.); and University of Chinese Academy of Sciences, Beijing 100049, China (H.H., Y.L.)

ORCID ID: 0000-0002-0622-4043 (G.L.).

The precise control of gene regulation, and hence, correct spatiotemporal tissue patterning, is crucial for plant development. Plant microRNAs can constrain the expression of their target genes at posttranscriptional levels. Recently, microRNA396 (miR396) has been characterized to regulate leaf development by mediating cleavage of its GROWTH-REGULATING FACTOR (GRF) targets. miR396 is also preferentially expressed in flowers. However, its function in flower development is unclear. In addition to narrow leaves, pistils with a single carpel were also observed in miR396 overexpression plants. The dramatically reduced expression levels of miR396 targets (GRF1, GRF2, GRF3, GRF4, GRF7, GRF8, and GRF9) caused pistil abnormalities, because the miR396-resistant version of GRF was able to rescue miR396-overexpressing plants. Both GRF and GRF-INTERACTING FACTOR (GIF) genes are highly expressed in developing pistils, and their expression patterns are negatively correlated with that of miR396. GRF interacted with GIF to form the GRF/GIF complex in plant cell nucleus. miR396 suppressed the expression of GRF genes, resulting in reduction of GRF/GIF complex. gif triple mutant displayed normal pistils, whereas gif single mutant produced abnormal pistils, which was a phenocopy of 35S:MIR396a/grf5 plants. GRF and GIF function as cotranscription factors, and both are required for pistil development. Our analyses reveal an important role for miR396 in controlling carpel number and pistil development via regulation of the GRF/GIF complex.

The precise spatial and temporal expression of regulatory genes that control tissue patterning and cell fate is important for plant development. Misexpression of certain key regulatory genes causes developmental abnormalities in plants. There is increasing evidence that small RNA molecules are important participants in the control of gene expression, providing sequence specificity for targeted regulation of key developmental factors at the posttranscriptional level. MicroRNAs (miRNAs) are 21- to 24-nucleotide noncoding RNAs that negatively regulate gene expression by pairing with their target mRNAs. They are produced from primary miRNAs that are transcribed from MIRNA genes. After the miRNA duplexes are released from the nucleus, mature miRNAs are recruited into an RNA-induced silencing complex associated with ARGONAUTE (AGO) proteins, where they suppress target mRNAs by complementary matching for cleavage and/or translational repression (Reinhart et al., 2002; Carrington and Ambros, 2003; Bartel, 2004; Brodersen et al., 2008; Lanet et al., 2009).

Several plant miRNAs have been shown to function in plant development. The lack of miRNA processing protein(s) can cause severe developmental phenotypes. For example, the weaker dicer-like1 (dcl1) alleles produce various aberrant morphological phenotypes, including extra whorls of stamens, an indefinite number of carpels, female sterility, altered ovule development, and reduced plant height, indicating that miRNA metabolism is essential for normal plant development (Schauer et al., 2002). ago1 null mutants exhibit morphological defects similar to those of dcl1, hua enhancer1, and hyponastic leaves1 (hyll) mutants (Vaucheret et al., 2004). The specific functions of miRNAs in floral development have been characterized. For example, Arabidopsis (Arabidopsis thaliana) microRNA160a (miR160a) mutant produced floral organs in carpels (Liu et al., 2010). Overexpression of miR164 led to flowers with fused sepals, which resembled the flowers of its target mutants, cuc1cuc2 (for cup-shaped cotyledon1cup-shapedcotyledon2; Mallory et al., 2004). Enhanced expression of the miR164-resistant version of mCUC1 resulted in flowers with more petals than those of the wild type (Baker et al., 2005).

Plants ectopically expressing miR166 showed extreme fasciation of the inflorescence meristem and a reduced or filamentous gynoecium (Kim et al., 2005; Williams et al., 2005). Constitutive expression of miR159 or miR167, which led to reduced expressions of their target genes (MYB33 and MYB65; Auxin Responsive Factor6 [ARF6] and ARF8), caused male sterility in Arabidopsis (Achard et al., 2004; Millar and Gubler, 2005; Ru et al., 2006; Wu...
et al., 2006). Elevated miR172 accumulation resulted in floral organ identity defects similar to those in its target gene mutant (apatala2; Aukerman and Sakai, 2003; Chen, 2004).

GROWTH-REGULATING FACTOR (GRF) genes are a class of plant-specific transcription regulators. In Arabidopsis, there are nine GRF genes that can be divided into five subfamilies: group I (GRF1 and GRF2), group II (GRF3 and GRF4), group III (GRF5 and GRF6), group IV (GRF7 and GRF8), and group V (GRF9; Kim et al., 2003). Among them, GRF1, GRF2, GRF3, GRF4, GRF7, GRF8, and GRF9 are the direct targets of miR396 (Jones-Rhoades and Bartel, 2004). It has been revealed that miR396 is involved in leaf development by controlling the levels of its GRF targets (Liu et al., 2009; Yang et al., 2009; Rodriguez et al., 2010; Wang et al., 2011; Debernardi et al., 2012). A GRF protein consists of two regions, the QLQ and WRC domains. The QLQ domain is responsible for protein interaction, while the WRC domain comprises a functional nuclear localization signal and a zinc-finger motif that functions in DNA binding (Kim et al., 2003).

GRF genes are involved in regulating leaf growth and morphology (Kim et al., 2003; Horiguchi et al., 2005). The GRF-INTERACTING FACTOR1 (GIF1) protein was identified to interact with GRF1 as a transcription coactivator to regulate leaf development (Kim and Kende, 2004). Horiguchi et al. (2005) revealed that both GRF5 and GRF9 interact with GIF1 to regulate leaf development. GIF1 contains two domains: the SNH and QG domains. The SNH domain is responsible for the interaction with the QLQ domain of GRF. The GIF gene family has three members, GIF1, GIF2, and GIF3, which have overlapping functions in determining organ (leaf and petal) size (Kim and Kende, 2004; Lee et al., 2009).

Another group (Hewezi et al., 2012) revealed the functions of miR396 in reprogramming root cells during infection by a parasitic cyst nematode. Here, we demonstrate that the products of all seven GRF targets can interact with GIFs that may function as cotranscription factors. Overexpression of miR396 caused reduced expressions of GRF genes, which disrupted the formation of the GRF/GIF complex, leading to pistil anomalies. These results indicate that miR396-directed regulation is critical for pistil development.

RESULTS

Overexpression of miR396 Resulted in Aberrant Pistils

Two MIR396 gene-encoding loci (MIR396a and MIR396b) have been identified in Arabidopsis. They are processed into two types of mature miR396s with only one nucleotide difference (Jones-Rhoades and Bartel, 2004). In our previous research, we found that miR396 was ubiquitously expressed in seedlings, roots, leaves, siliques, and inflorescences and that constitutive expression of miR396 caused narrow leaves by targeting GRF genes in Arabidopsis (Liu et al., 2009). Further investigation found that miR396-overexpressing plants produced flowers with various deformations. Wild-type flowers often contain four sepals, four petals, six stamens, and two fused carpels (Fig. 1A). In miR396-overexpressing plants, approximately 70% of flowers contained aberrant pistils, such as extremely bent pistils, unfused carpels, and single carpels (Fig. 1, B–D). The aberrant pistils formed into short siliques (Fig. 1E). The single-carpel siliques contained only one column of seeds (Fig. 1F), which accounted for approximately 65% of all siliques (Fig. 1G). The abnormal siliques resulted in lower fertility compared with that of the wild type (Fig. 1H).

Given the fact that increased levels of miR396 led to aberrant pistils, we asked what would happen when miR396 expression was repressed. To suppress the functions of both miR396a and miR396b, we used the Short Tandem Target Mimic (STTM) strategy (Yan et al., 2012) to construct STTM396-transgenic plants. Northern-blotting analysis indicated that miR396 was moderately decreased in the flowers of STTM396 plants (Supplemental Fig. S1A). However, the pistils and siliques of STTM396 plants were normal (Supplemental Fig. S1B).

miR396 Suppressed Expression of GRF Genes in Floral Organs

In Arabidopsis, there are nine GRF genes, seven of which are predicted to be targeted by miR396. The cleavage of six GRF genes (GRF1, GRF2, GRF3, GRF7, GRF8, and GRF9) has been validated experimentally (Jones-Rhoades and Bartel, 2004). We confirmed the cleavage of GRF4 in the predicted miR396 recognition site by 5’ RACE experiments (Supplemental Fig. S2A).

We compared the transcript levels of GRF genes in flowers among the wild type, 35S:MIR396a, and STTM396 plants; the level of miR396 was negatively correlated with those of its GRF targets (Fig. 2A). Unexpectedly, two nontargeted GRF genes were affected differently by miR396. Like the other targeted GRF genes, the level of GRF6 transcripts was negatively correlated with that of miR396. By contrast, the transcript level of GRF5 was not influenced by miR396. The levels of GRF transcripts and miR396 were further examined in different floral organs of the wild type. The lowest level of miR396 and the highest levels of GRF transcripts were in the pistil (Fig. 2B). Considering the high frequency of altered pistils in miR396-overexpressing plants, we investigated the levels of GRF transcripts and miR396 in the pistils of flowers at three different developmental stages (Fig. 2C). There were relatively high transcript levels of GRF genes at stages 10 and 13, but low levels at stage 15. By contrast, the transcript levels of both MIR396a and MIR396b were relatively low at stages 10 and 13, but high at stage 15. Taken together, these results indicated that miR396 may constrain the expression of GRF genes.

To confirm the direct regulation of GRF genes by miR396 in planta, we performed transient coexpression assays in Nicotiana benthamiana. We generated two types of constructs for both GRF7 and GRF9, the miR396-sensitive constructs 35S:GRF7 and 35S:GRF9 and the
miR396-resistant constructs 35S:mGRF7 and 35S:mGRF9. The miR396-resistant version of mGRF contained three silent mutations within the miR396-complementary domain of the GRF genomic clone, thereby increasing the number of mismatches between miR396 and mGRF without altering the amino acid sequence of the encoded GRF protein (Supplemental Fig. S2B). After 3 d of coexpression in N. benthamiana, RNA was extracted and the transcript abundances of GRF7 and GRF9 were analyzed by real-time quantitative reverse transcription (qRT)-PCR. The mRNA levels of miR396-resistant mGRF7 or mGRF9 were not affected by coexpression with MIR396a. However, mRNA levels of the miR396-sensitive GRF7 and GRF9 were significantly decreased when coexpressed with MIR396a (Fig. 2D). These findings suggested that miR396 directly mediates the cleavage of GRF genes in planta.

miR396-Resistant mGRF7 or mGRF9 Rescued miR396 Transgenic Plants

Analyses of the expression patterns of GRF genes showed that all nine GRF genes are expressed in roots, upper stems, and shoot tips containing the shoot apical meristem and flower buds, as well as in mature flowers (Kim et al., 2003). Because GRF genes are suppressed by miR396, we expected that GRF mutants could phenocopy miR396-overexpressing plants. We obtained six GRF single mutants (Supplemental Fig. S3A), grf1, grf3, grf4, grf7, grf8, and grf9, all of which produced normal siliques (Supplemental Fig. S3B). The grf1grf2grf3 triple mutants (ecotype Wassilewskija background; Kim et al., 2003) have small leaves, but normal floral organs and fertility. The leaves of the grf7 single mutant were reported to be smaller than those of the wild type (Kim et al., 2012), implying that group IV GRF genes may play a dominant role. Therefore, we constructed a grf7grf8 double mutant and a grf7grf8grf9 triple mutant. These mutants produced normal siliques (Supplemental Fig. S3B). In flowers of miR396-overexpressing plants, all GRF genes except for GRF5 were down-regulated (Fig. 2A). We could not investigate the individual functions of the GRF genes because of the 8-fold redundancy and their overlapping expression patterns. It was also very difficult to obtain an octuple mutant for the eight down-regulated GRF genes because of their close linkages on chromosomes.

To investigate whether reduced expressions of GRF genes caused the aberrant pistils of miR396-overexpressing plants, we conducted functional complementation tests. Each of four GRF constructs (35S:GRF7, 35S:mGRF7,
35S:GRF9, and 35S:mGRF9) was transformed into Arabidopsis wild-type plants. All transgenic plants produced normal siliques (Supplemental Fig. S3C). When 35S:GRF7 or 35S:GRF9 plants were crossed with 35S:MIR396a plants, their progenies (35S:GRF7/35S:mIR396a or 35S:GRF9/35S:mIR396a) still formed abnormal pistils and single-carpet siliques, although there were smaller proportions of abnormal siliques. By contrast, when 35S:mGRF7 or 35S:mGRF9 plants were crossed with 35S:MIR396a plants, their progenies (35S:mGRF7/35S:miR396a or 35S:mGRF9/35S:miR396a) developed normal siliques (Fig. 3A). We further quantified the transcript levels of GRF7 and GRF9 in F1 progenies (Fig. 3B). As expected, the level of GRF7 transcripts was dramatically decreased in 35S:GRF7/35S:miR396a plants compared with that in 35S:GRF7/wild-type plants. By contrast, the levels of GRF7 transcripts in 35S:mGRF7/35S:miR396a plants were similar to that in 35S:mGRF7/wild-type plants. A similar case was also observed for GRF9. Therefore, miR396-resistant mGRF7 and mGRF9, but not miR396-sensitive GRF7 and GRF9, were sufficient to recover 35S:MIR396a. Our results suggested that the reduced expressions of GRF genes were responsible for the pistil abnormalities of miR396-overexpressing plants.

GRF Interacted with GIF as Cotranscription Factors

Previous studies demonstrated that GRF1 and GIF1 function as cotranscription factors in regulating leaf growth and morphology in Arabidopsis (Kim and Kende, 2004). In Arabidopsis, there are two homologs (GIF2 and GIF3) of GIF1. To determine whether each GRF protein can interact with each GIF protein, we used yeast (Saccharomyces cerevisiae)-two hybridization assays to investigate their interactions. As shown in Figure 4A, GIF1 strongly interacted with seven GRFs, but only weakly interacted with GRF4 and GRF7. By contrast, both GIF2 and GIF3 strongly interacted with all GRFs except for GRF9.

Next, we used bimolecular fluorescence complementation (BiFC) assays to verify these protein interactions in planta. The N-terminal fragment of yellow fluorescent protein (nYFP) was individually ligated with GRF4, GRF7, and GRF9 to produce GRF4-nYFP, GRF7-nYFP, and GRF9-nYFP, respectively. The GIF1, GIF2, and GIF3 proteins were individually fused with the C-terminal fragment of YFP (cYFP). When GIF1-cYFP was transiently coexpressed with GRF9-nYFP, strong YFP fluorescence was visible in the nucleus of epidermal cells in N. benthamiana leaves (Fig. 4B), whereas no YFP fluorescence was detected in negative controls (GIF1-cYFP coexpressed with nYFP or cYFP coexpressed with GRF9-nYFP, Supplemental Fig. S4). Similar results were observed for coexpression of GIF2-cYFP with GRF7-nYFP and GIF3-cYFP with GRF4-nYFP (Fig. 4B).

To further confirm whether GRF and GIF form protein complex in plant cells, we performed communoprecipitation assays (Fig. 4C). GRF and GIF were transiently
coexpressed in tobacco (Nicotiana benthamiana) leaves. The total proteins were incubated with Flag antibody and A/G-agarose beads and then separated on SDS-PAGE for immunoblotting with Myc antibody. In agreement with the results in BiFC, GRF and GIF exist in the same protein complex. Taken together, our results suggested that GRFs and GIFs function as cotranscription factors.

Spatiotemporal Expression of GRF and GIF Genes in Flowers

Our results demonstrated that GRF proteins can physically interact with GIF proteins. To serve as cotranscription factors in flowers, GRFs and GIFs must have the same spatiotemporal expression patterns. To determine their expression patterns, Arabidopsis was transformed with GUS fusion constructs for each of these 12 genes (Fig. 5A). In flowers, the GRF3 promoter drove GUS expression in the receptacle and the GRF8 promoter drove GUS expression in the anther. The remaining seven GRF gene promoters and three GIF gene promoters were mainly activated in the pistil, although they showed somewhat different spatial expression patterns.

We further analyzed the transcript levels of GIF genes in sepal, petals, stamens, and pistils. Like GRF genes (Fig. 2, B and C), the highest levels of GIF transcripts were in the pistil (Fig. 5B) and their expressions in the pistil decreased at later stages of flower development (Fig. 5C).

Suppression of GRF Genes by miR396 Caused the Reduction of GRF/GIF Complex

Because GRF genes are posttranscriptionally regulated by miR396, we expected that increased accumulation of...
miR396 would reduce the abundance of GRF/GIF complexes. To confirm our hypothesis, transient expression assays were conducted in N. benthamiana leaves (Fig. 6). When GIF2-cYFP and GRF7-nYFP were coexpressed with miR396a, only a few epidermal cells displayed visible YFP fluorescence. As a negative control, when they were coexpressed with miR395a, which cannot recognize the GRF7 gene, many cells showed YFP fluorescence. When GIF2-cYFP and mGRF7-nYFP were coexpressed with miR396a, most cells showed strong YFP fluorescence. These results indicated that the suppressed GRF expression reduces the formation of GRF/GIF complex.

**Figure 5.** Tissue-specific expression of GRF and GIF genes. A, Inflorescence staining of indicated genes. B, Expression of GIF genes in sepal, petal, stamen, and pistil at floral stage 13. C, Expression of GIF genes in pistils at the indicated floral stage.
Phenotypes of GIF Triple Mutant gif1/gif2/gif3 Were Similar to Those of 35S:MIR396a/grf5 Plants

Because GRF and GIF function as cotranscription factors, we expected that the GIF mutants would phenocopy miR396-overexpressing plants. Previous studies (Kim and Kende, 2004) revealed that the gif1 mutant produces narrow leaves similar to those of 35S:MIR396a. We examined the siliques of gif1 mutants and found that all of them contained two carpels, but they were significantly shorter than wild-type siliques. We speculated that GIF1 would be functionally redundant with the other two GIF genes. To confirm our hypothesis, we constructed gif1/gif2, gif1/gif3, gif2/gif3, and gif1/gif2/gif3 mutants by crossing gif single mutants (Supplemental Fig. S5A). Compared with the siliques of the gif1 mutant, those of the gif1/gif2 and gif1/gif3 mutants were shorter, but those of the gif2/gif3 mutant were of a similar length. By contrast, the siliques of triple mutants were shorter than those of double mutants (Supplemental Fig. S5B). The gif1/gif2/gif3 triple mutant formed bent siliques, single-carpel siliques, and unfused-carpel siliques (Fig. 7, A–D). However, there were markedly fewer single-carpel siliques in gif1/gif2/gif3 mutants than in 35S:MIR396a plants (Fig. 7E). In addition, gif1/gif2/gif3 mutants showed very low fertility with no more than 20 seeds per plant, because most siliques did not contain seeds. This differed from 35S:MIR396a plants, in which only about 10% of siliques did not contain seeds.

Although eight GRF genes showed reduced transcript levels in 35S:MIR396a plants, GRF5 transcripts were not affected by miR396, which may have contributed to the higher fertility of 35S:MIR396a plants compared with gif1/gif2/gif3 mutants. To explore this idea, the grf5 mutant was crossed with 35S:MIR396a plants and 35S:MIR396a/grf5 plants were obtained by screening F2 plants. As expected, 35S:MIR396a/grf5 plants displayed lower fertility and had fewer single-carpel siliques than did 35S:MIR396a plants, indicating that 35S:MIR396a/grf5 plants phenocopied gif1/gif2/gif3 mutant plants (Fig. 7, D and E).

DISCUSSION

miRNAs play a key role in regulating plant development, which can be inferred from the developmental defects in dc11, hy1, serrate, and ago1 mutants and from analyses of plants overexpressing various miRNAs (e.g. miR160, miR164, miR166, and miR319, etc.). Here, we demonstrated that overexpression of miR396 results in morphological defects in floral organs. Our results
reveal the function of miR396 in reducing the formation of the GRF/GIF complex, which regulates pistil development.

Functional Redundancies of GRF Family Members

Previously, we showed that overexpression of miR396 results in narrow rosette leaves and reduced expressions of its GRF target genes (Liu et al., 2009). Here, our results indicate that elevated miR396 also causes floral abnormalities by suppressing the expressions of its GRF target genes. The Arabidopsis genome contains nine GRF genes, all of which are expressed in leaves and flowers. However, only grf5 and grf7 single mutants produced leaves slightly smaller than those of wild-type plants (Horiguchi et al., 2005; Kim et al., 2012), whereas the other grf single mutants displayed no developmental abnormalities. The grf1/grf2/grf3 triple mutants produced small leaves and coryledons, indicating that GRF1, GRF2, and GRF3 participate redundantly in controlling leaf cell number (Kim et al., 2003). Previous investigations confirmed that overexpression of GRF1, GRF2, or GRF5 can increase the leaf size area (Kim et al., 2003; Horiguchi et al., 2005). We found that plants overexpressing GRF7 or GRF9 showed slightly enlarged leaves (Supplemental Fig. S6), but grf7/grf8/grf9 mutants produced leaves similar to those of grf7 single mutants, suggesting their overlapping functions in regulating leaf development. Our results revealed that the down-regulation of GRF genes is responsible for the aberrant silique of miR396-overexpressing plants, because the miR396-resistant version of GRF could recover their silique phenotypes. Both grf1/grf2/grf3 and grf7/grf8/grf9 mutants produced normal siliques, indicating redundant functions of these GRF genes in regulating floral development. Although GRF5 is not the target of miR396, a grf5 mutation aggravated the abnormalities in leaves and siliques of 35S:MIR396a plants (Fig. 7D; Supplemental Fig. S7), indicating its redundant functions with other GRF genes. Interestingly, the introduction of grf5 into 35S:MIR396a plants reduced the number of single-carpel siliques. Meanwhile, we also observed that it also led to nearly sterility. It seems that the single carpel ensures the production of necessary seeds. Further investigation is required to reveal the underlined mechanism. In addition, all of the GRF genes showed overlapping expression patterns in the flower, with their highest expression levels in the pistil. Taken together, these results showed that GRF genes function redundantly in regulating plant development.

GRF and GIF Coregulate Pistil Development

GRF proteins contain two conserved domains, QLQ and WRC, in their N-terminal region. The QLQ domain is very similar to the N-terminal part of the SWI2/SNF2 protein that interacts with another component of the SWI2/SNF2 chromatin-remodeling complex in yeast (Trech et al., 1995). The WRC domain consists of a functional nuclear localization signal and a DNA-binding motif. The C-terminal regions of GRFs have common features of transcription factors and are required for their transcription activation activities because N-terminal-truncated GRFs lose their transactivation functions (Fig. 4A). GIF proteins contain a SNH domain and a QG domain, which are similar to domains in the SYT protein, a transcriptional coactivator in human (Brett et al., 1997). The SYT protein can interact with SWI2/SNF2-chromatin remodeling proteins, which may regulate transcription via chromatin modification (Thaete et al., 1999; Kato et al., 2002; Aizawa et al., 2004). Similarly, our results indicated that GIF proteins can interact with GRF proteins in both yeast and plant cells. Thus, similar to the interaction between SYT and SWI2/SNF2 proteins, GIF interacts with GRF to influence the transcriptions of downstream target genes.

miR396-overexpressing plants, in which all of the GRF genes except for GRF5 were dramatically down-regulated, produced abnormal pistils. The spatiotemporal expression patterns of GRF genes in the flower were very similar to those of GIF genes, and both were expressed at relatively high levels in the pistil. The combination of their expression patterns and their interactions in the plant cell nucleus implied that GRF and GIF may coregulate pistil development. Our results revealed that each GIF protein can interact with almost all of the GRF proteins, implying that one GIF modulates the functions of multiple GRFs. That explains why grf1 single mutants, but not grf single mutants, displayed short si- lique. We also observed that siliques of 35S:MIR396a/grf5 were shorter than those of 35S:MIR396a (Fig. 7E). The siliques of gif double mutants (gif1/gif2 and gif1/gif3) were shorter than those of gif1 mutants, and the siliques of gif1/gif2/gif3 triple mutants were shorter than those of double mutants (Supplemental Fig. S7B). Both 35S:MIR396a/grf5 and gif1/gif2/gif3 caused short and almost completely sterile siliques as well as single-carpel siliques, suggesting that silique development is GRF and GIF dose dependent.

Proper Regulation of GRF Genes by miR396 Is Crucial for Plant Development

Several miRNAs have been shown to function in regulating floral development (Achard et al., 2004; Chen, 2004; Mallory et al., 2004; Williams et al., 2005; Wu et al., 2006), and all of these miRNAs are conserved across plant species. The misexpression of these miRNAs followed by misexpression of their targets can cause abnormal development of floral organs, suggesting that a balance between these miRNAs and their targets is required for floral development. Similarly, we demonstrated that overexpression of miR396 mediated the down-regulation of their GRF targets, resulting in abnormal floral organs. In wild-type flowers, all nine GRF genes were highly expressed at an early stage of pistil development, and their abundance decreased as the siliques mature. By contrast, both MIR396a and MIR396b genes were expressed at low levels in young siliques and their abundance increased as the siliques
matured. The inverse correlation between miR396 and its targets implied that miR396 constrains the expression of its target genes. However, the balance between miR396 and its targets was disrupted in flowers of miR396-overexpressing plants, and its target genes were dramatically down-regulated in the flowers, compared with those in wild-type flowers. The altered expressions of GRF genes were responsible for the deformed pistils in 35S:MIR396a plants. When 35S:MIR396a plants were crossed with 35S:GRF7/GRF9 plants, their progenies, 35S:GRF7/GRF9/35S:MIR396a, displayed phenotypes identical to those of 35S:MIR396a plants. By contrast, 35S:mGRF7/GRF9 restored 35S:MIR396a plants to the wild type. Therefore, the right amount of GRF levels is required for pistil development. A similar case was also observed in tobacco, where overexpression of miR396 caused aberrant pistils via down-regulation of GRF targets (Yang et al., 2009; Baucher et al., 2013). We also observed that the narrow leaf phenotypes were always linked with aberrant pistils and elevated GRF expression could rescue both defects, implying that the miR396/GRF cascade regulates the development of both leaf and flower. These results revealed that appropriate regulation of GRF genes by miR396 is necessary for plant development.

Although we reveal that miR396 affects the development of pistils by regulation of GRF genes, it is still unclear which developmental processes were linked to the pistil abnormalities. As shown in Figure 5A, both GRF and GIF genes were expressed in the early pistil developmental stages. It is likely that the GRF/GIF complexes control the expression of genes involved in early pistil development. Further investigation into the detailed expression patterns of GRF and GIF genes and to establish the direct targets regulated by the GRF/GIF complexes are required to better understand the mechanism of pistil formation.

**Materials and Methods**

**Plant Materials**

Arabidopsis (Arabidopsis thaliana) ecotype Columbia was used for all experiments. The generation of 35S:MIR396a plants were described previously (Liu et al., 2009). Plants were grown in long photoperiods (16-h light/8-h dark) or in short photoperiods (8-h light/16-h dark) at 23°C.

**Real-Time qRT-PCR Experiments**

One microgram total RNA extracted using the Trizol reagent (Invitrogen) was used for oligo(dT)18 primed complementary DNA (cDNA) synthesis according to the reverse transcription protocol (Fermentas). The resulting cDNA was subjected to relative quantitative PCR using a SYBR Premix Ex Taq kit (TaKaRa) on a Roche LightCycler 480 real-time PCR machine, according to the manufacturer’s instructions. For each reported result, at least three independent biological samples were subjected to minimum of three technical replicates. The results were normalized to ACTIN2. The qRT-PCR primers for nine GRFs were described previously (Rodriguez et al., 2010). The other qRT-PCR primers used are listed in Supplemental Table S1.

**Plasmid Construction**

The pOCA30 binary plasmid was used for an expression vector. For overexpression, the genome sequence for each gene was cloned into the pOCA30 vector. The miRNA target motif in GRF7 or GRF9 was altered, introducing synonymous mutations in a cloned GRF7 or GRF9 wild-type genomic fragment.

**Yeast (Saccharomyces cerevisiae) Assays**

For yeast-two hybridization assay, all the N-terminal truncated GRFs containing the Qldand WRC domains were cloned into pGBK7, and the full-length Gif genes were cloned into pGADT7. The N-terminal sequences of the GRFs for yeast two-hybrid assay can be found according to the primers provided in Supplemental Table S1. For transactivation assays, the full-length GRF and Gif cDNAs were cloned into pGBK7 and introduced into yeast cells. Growth was determined as described in the Yeast Two-Hybrid System User Manual (Clontech). Primers used for the vector construction were listed in Supplemental Table S1. Experiments were repeated three times.

**Agrobacterium tumefaciens Infiltration in Nicotiana benthamiana**

Plasmids were transformed into A. tumefaciens strain EHA105. Agrobacterial cells were infiltrated into leaves of N. benthamiana. For miRNA/GRF co-coinfiltration experiments, equal volumes of an A. tumefaciens culture containing 35S:MIR396a (optical density at 600 nm [OD600] = 1.75) and 35S:mGRF7/GRF9 (OD600 = 0.25) were mixed before infiltration into N. benthamiana leaves. After infiltration, plants were placed at 24°C for 72 h before RNA extraction.

For BiFC assays, full-length coding sequences of Arabidopsis GRF4, GRF7, GRF9, GIF1, GIF2, and GIF3 were cloned into the binary nYFP or cYFP vector. A. tumefaciens strains transformed with indicated nYFP or cYFP vector were incubated, harvested, and resuspended in infiltration buffer (0.2 mS aceto-syringone, 10 mM MgCl2, and 10 mM MES; pH 5.6) to identical concentrations (OD600 = 0.5). For miRNA/GRF/GIF interaction test, equal volumes of an A. tumefaciens culture containing 35S:MIR396a or 35S:MIR396a (OD600 = 2.5), 35S:mGRF7-nYFP (OD600 = 0.5), and 35S:GIF2-cYFP (OD600 = 0.5) were mixed before infiltration into N. benthamiana leaves. After infiltration, plants were placed at 24°C for 48 h before observation.

**Coimmunoprecipitation Assay**

Flag-GRF-nYFP and Myc-GIF-cYFP (or Myc-cYFP) vector were transiently coexpressed in N. benthamiana leaves. Infected leaves were harvested 48 h after infiltration and used for protein extraction. Flag-fused GRF was immunoprecipitated using Flag antibody, and the coimmunoprecipitated proteins were then detected using Myc antibody.

**Scanning Electron Microscopy and GUS Assays**

For scanning electron microscopy analysis, siliques from flowers at stage 15 were separated, fixed, dehydrated, dried, coated with gold-palladium, and then photographed.

For promoter-GUS constructs of GRF and GIF genes, about 2-kb upstream promoter regions were amplified and fused with the GUS gene. The primers were listed in Supplemental Table S1. Transgenic plants were subjected to GUS staining as described previously (Liang et al., 2010).

Sequence data from this article can be found in the GenBank/EMBL database under accession numbers MIR396a (AT5G06060), MIR396b (AT5G35407), GFR1 (AT5G22840), GFR2 (AT4G37740), GFR3 (AT3G36400), GFR4 (AT3G52910), GFR5 (AT3G13960), GFR6 (AT2G06200), GFR7 (AT5G35660), GFR8 (AT4G24150), GFR9 (AT2G45840), GIF1 (AT5G28640), GIF2 (AT1G01160), GIF3 (AT1G00850), and ACT2 (AT3G18780). The transfer DNA insertion mutants used in this article include gfr1 (SALK_069399C), gfr3 (SALK_020766C), gfr4 (SALK_077829C), gfr5 (SALK_086597C), gfr7 (CS879693), gfr8 (CS804312), gfr9 (SALK_140746C), gif1 (SALK_150407), and gif3 (SALK_052744).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1. STTM396 plants.**
Supplemental Figure S2. Cleavage of GRF4 and modification of GRF7 and GRF9.

Supplemental Figure S3. Siliques of grf mutants and GRF transgenic plants.

Supplemental Figure S4. Negative controls for BiFC assays.

Supplemental Figure S5. grf mutants.

Supplemental Figure S6. Leaf area of third leaves of wild-type, 35S:MR396a, and various GRF transgenic plants.

Supplemental Figure S7. Rosette leaves of wild-type, 35S:MR396a, 35S:MR396a/grfgrf, and grf/grf/grf plants.

Supplemental Table S1. Primers used in this article.

ACKNOWLEDGMENTS

We thank the editor and two anonymous reviewers for their constructive comments, which helped us to improve the manuscript, the Arabidopsis Resource Center at the Ohio State University for the transfer DNA insertion mutants, and Yanhui Zhao (Kunming Institute of Botany, Chinese Academy of Sciences) for scanning electron microscope assistance.

Received July 18, 2013; accepted November 25, 2013; published November 27, 2013.

LITERATURE CITED


