Molecular Mechanism of microRNA396 Mediating Pistil Development in Arabidopsis[1][W]

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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* single mutant displayed normal pistils, whereas *gif* triple mutant *gif1/gif2/gif3* 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* (*hyl1*) 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

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et al., 2006). Elevated miR172 accumulation resulted in floral organ identity defects similar to those in its target gene mutant (*apetala2*; 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 *MIR396*a 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

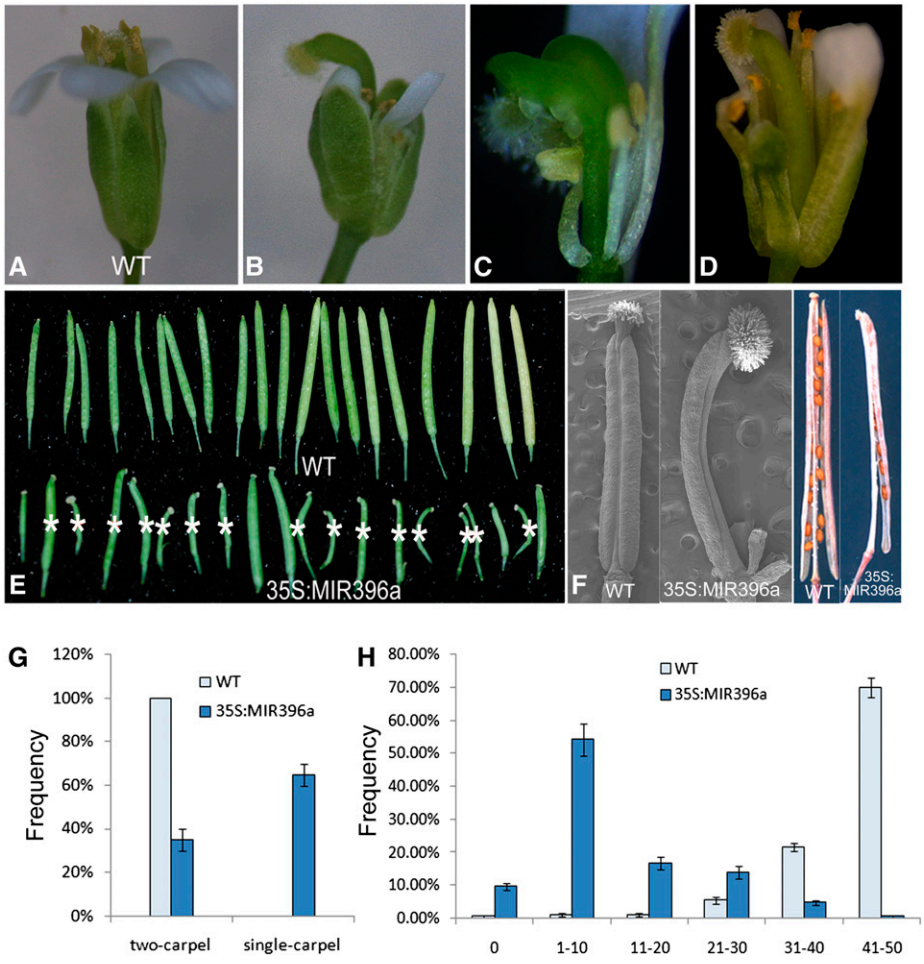

Figure 1. Phenotypes of *35S:MIR396a* plants. A, Wild-type flower. B to D, Bent pistil (B), unfused carpels (C), and single-carpel pistil (D) in the flowers of *35S:MIR396a* plants. E, First 20 siliques in wild-type and *35S:MIR396a* plants. The white asterisk indicates the single-carpel siliques. F, Siliques at stage 15 (left) and mature siliques (right). G, Percentage of siliques containing single carpel or two carpel. H, Percentage of siliques with the indicated number of seeds. Three individuals for each genotype were used for silique analysis. Thirty siliques for each individual were analyzed.

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*,

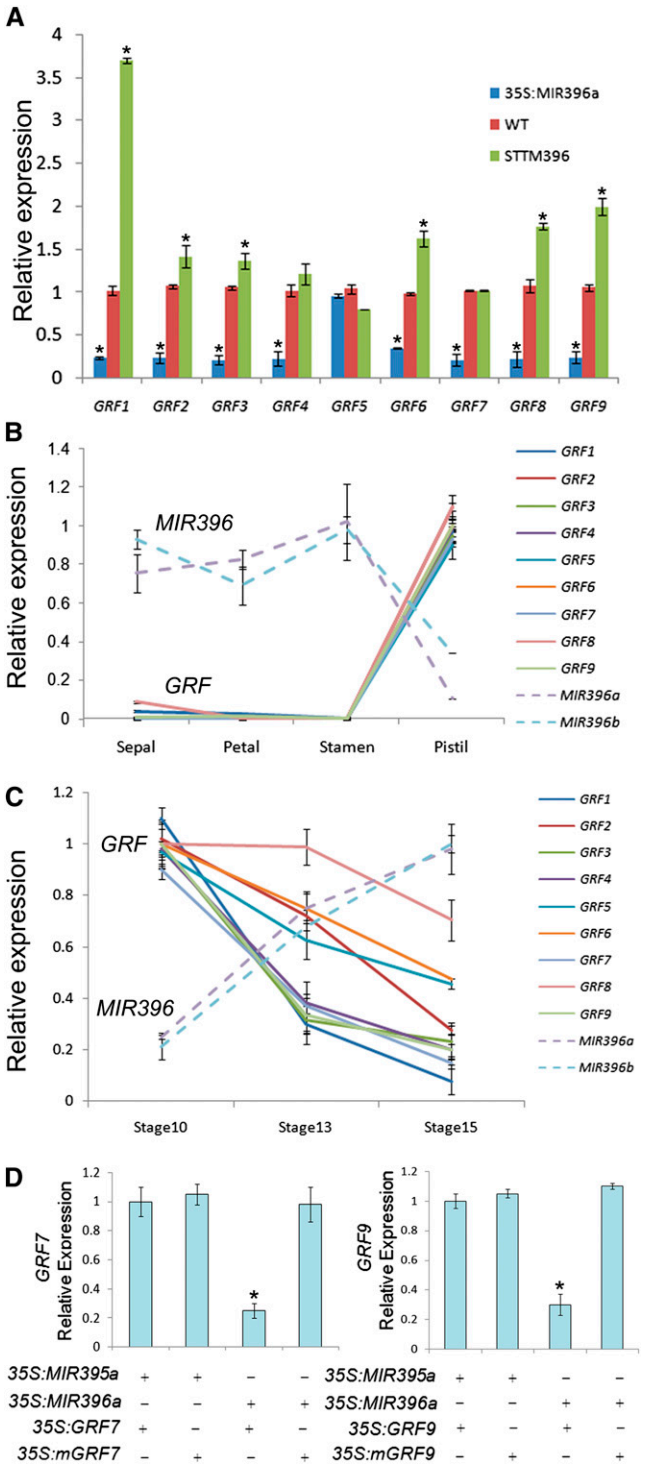

Figure 2. Regulation of *GRF* genes by miR396. A, Transcript levels of *GRF* genes in flowers. B, Transcript levels of *MIR396* and *GRF* genes in sepal, petal, stamen, and pistil at floral stage 13. C, Transcript levels of *MIR396* and *GRF* genes in pistil at the indicated floral stages. D, Coexpression of various combinations of *MIRNA* and *GRF* expression constructs in *N. benthamiana*. A to D, Error bars represent SE for three independent experiments. The values marked by an asterisk are significantly different from the control values ($P < 0.01$; $n = 3$).

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-carpel 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 coimmunoprecipitation assays (Fig. 4C). GRF and GIF were transiently

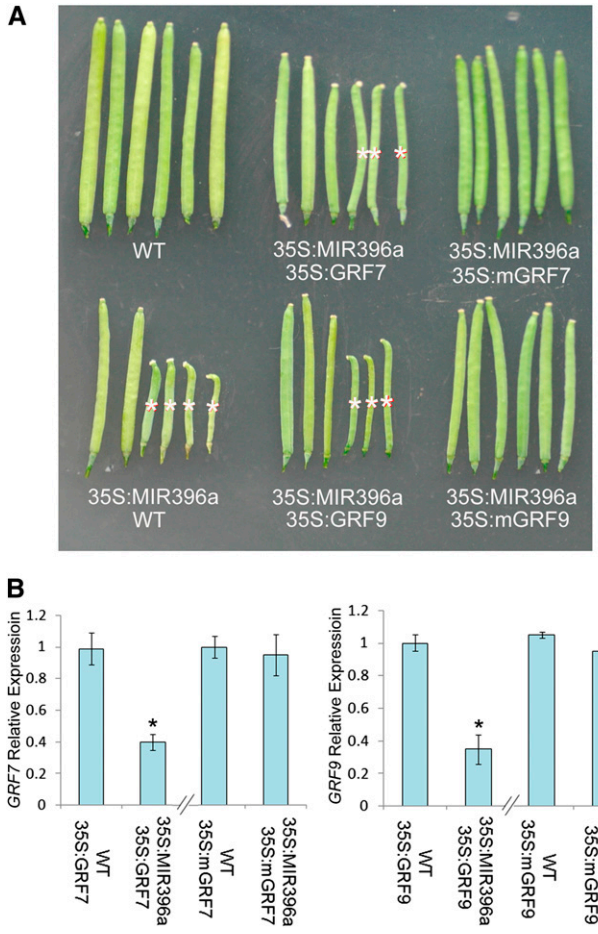

Figure 3. Rescue of *35S:MIR396a* by *mGRF*. A, Six representative siliques were presented for each plant. The white asterisk indicates the single-carpel siliques. B, Expression of GRF7 and GRF9. Flowers of F1 progenies for indicated two parental plants were used for expression analysis. Error bars represent SE for three independent experiments. The values marked by an asterisk are significantly different from the control values ($P < 0.01$; $n = 3$).

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 sepals, 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

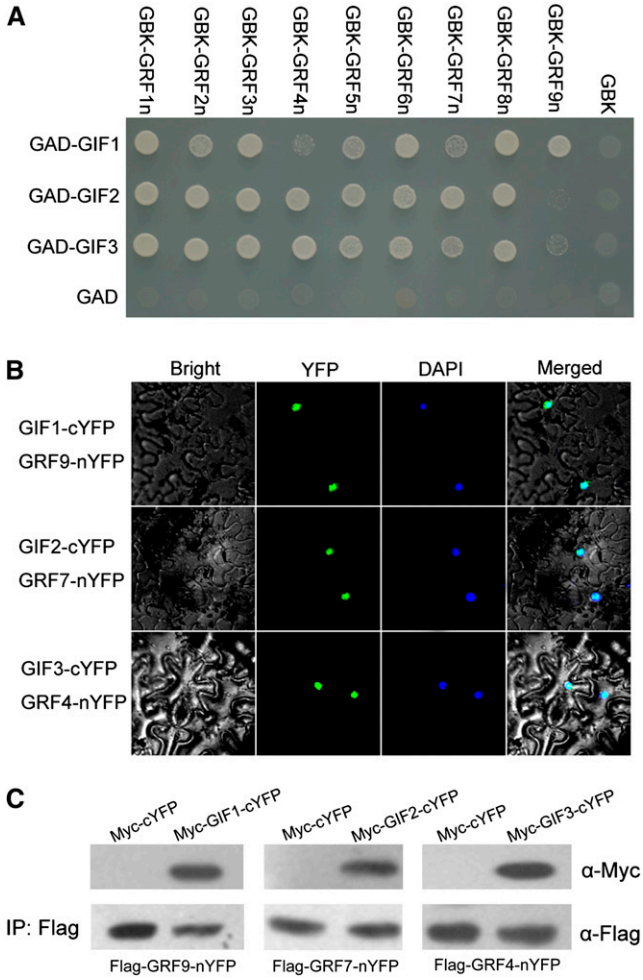

Figure 4. Interaction of GRFs and GIFs. A, Yeast two-hybrid assays. Interaction was indicated by the ability of cells to grow on synthetic dropout medium lacking Leu/Trp/His/Ade. N-terminal truncated GRFs and full-length GIFs were cloned into pGBKT7 and pGADT7, respectively. B, BiFC assays. Fluorescence was observed in nuclear compartments of *N. benthamiana* leaf epidermal cells; the fluorescence resulted from complementation of the N-terminal portion of YFP fused to GRF (GRF-nYFP) with the C-terminal portion of YFP fused to GIF (GIF-cYFP). C, CoIP assays. Flag-fused GRF-nYFP was immunoprecipitated using Flag antibody, and coimmunoprecipitated Myc-GIF-cYFP was then detected using Myc antibody.

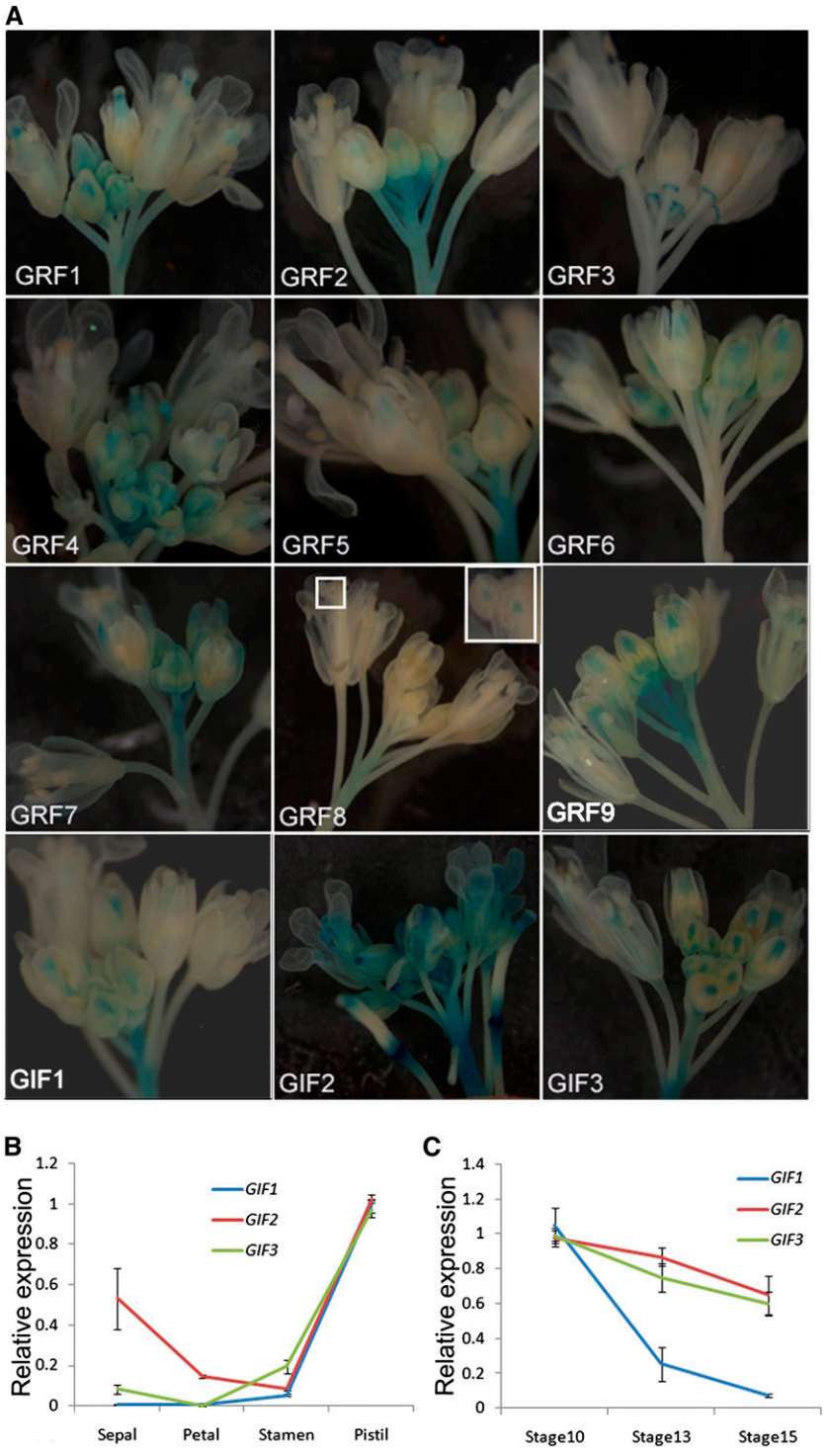

Figure 5. Tissue-specific expression of *GRF* and *GIF* genes. A, Inflorescence staining of indicated genes. B, Expression of *GIF* genes in sepal, petal, stamen, and pistil at floral stage 13. C, Expression of *GIF* genes in pistils at the indicated floral stage.

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.

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).

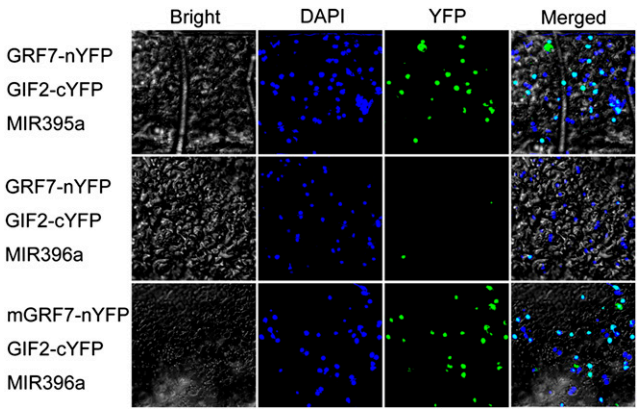

Figure 6. miR396 suppresses formation of the GRF/GIF complex. *MIRNA*, *(m)GRF7-nYFP*, and *GIF2-cYFP* constructs were coinfiltrated into tobacco epidermal cells as described in "Materials and Methods." *MIR395a* was used as a negative control.

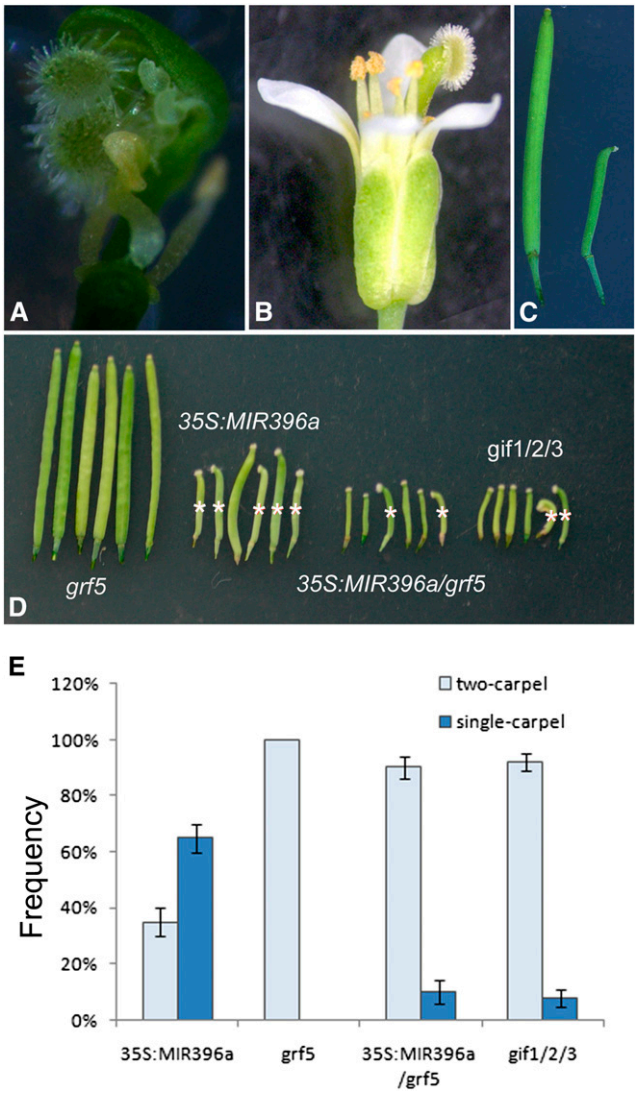

Figure 7. *gif1/gif2/gif3* triple mutants mimic *35S:MIR396a/grf5*. A, Pistil with unfused carpels in *gif1/gif2/gif3* mutants. B, Single-carpel pistil in the *gif1/gif2/gif3* flower. C, Normal siliques (left, wild type) and single-carpel silique (right, *gif1/gif2/gif3*). D, Representative siliques for the indicated plants. The white asterisk indicates the single-carpel siliques. E, Percentage of siliques containing single carpel or two carpel. Three individuals for each genotype were used for silique analysis. Thirty siliques for each individual were analyzed.

DISCUSSION

miRNAs play a key role in regulating plant development, which can be inferred from the developmental defects in *dcl1*, *hyl1*, *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/gif2/gif3* triple mutants produced small leaves and cotyledons, 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 surface 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 siliques 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 (Treich 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 *gif1* single mutants, but not *grf* single mutants, displayed short siliques. 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 QLQ and WRC domains were cloned into pGBKT7, 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 pGBKT7 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 coinfiltration experiments, equal volumes of an *A. tumefaciens* culture containing *35S:MIR396a* (optical density at 600 nm [OD600] = 1.75) and *35S:(m)GRF7/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 mM acetosyringone, 10 mM $MgCl_2$, 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:MIR395a* or *35S:MIR396*a (OD600 = 2.5), *35S:(m)GRF7-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) 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 data libraries under accession numbers *MIR396a* (AT2G10606), *MIR396b* (AT5G35407), *GRF1* (AT2G22840), *GRF2* (AT4G37740), *GRF3* (AT2G36400), *GRF4* (AT3G52910), *GRF5* (AT3G13960), *GRF6* (AT2G06200), *GRF7* (AT5G53660), *GRF8* (AT4G24150), GRF9 (AT2G45480), *GIF1* (AT5G28640), *GIF2* (AT1G01160), *GIF3* (AT4G00850), and *ACT2* (AT3G18780). The transfer DNA insertion mutants used in this article include *grf1* (SALK_069339C), *grf3* (SALK_026786), *grf4* (SALK_077829C), *grf5* (SALK_086597C), *grf7* (CS878963), *grf8* (CS804312), *grf9* (SALK_140746C), *gif1* (SALK_150407), *gif2* (CS851972), 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:MIR396a*, and various *GRF* transgenic plants.

Supplemental Figure S7. Rosette leaves of wild-type, *35S:MIR396a*, *35S:MIR396a/grf5*, and *gif1/gif2/gif3* plants.

Supplemental Table S1. Primers used in this article.

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