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miR319a targeting of *TCP4* is critical for petal growth and development in *Arabidopsis*

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In a genetic screen in a *drnl-2* background, we isolated a loss-of-function allele in *miR319a* (*miR319a*¹²⁹). Previously, *miR319a* has been postulated to play a role in leaf development based on the dramatic curled-leaf phenotype of plants that ectopically express *miR319a* (*jaw-D*). *miR319a*¹²⁹ mutants exhibit defects in petal and stamen development; petals are narrow and short, and stamens exhibit defects in anther development. The *miR319a*¹²⁹ loss-of-function allele contains a single-base change in the middle of the encoded miRNA, which reduces the ability of *miR319a* to recognize targets. Analysis of the expression patterns of the three members of the miR319 gene family (*miR319a*, *miR319b*, and *miR319c*) indicates that these genes have largely non-overlapping expression patterns suggesting that these genes have distinct developmental functions. *miR319a* functions by regulating the TCP transcription factors *TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24*; the level of RNA expression of these TCP genes is down-regulated in *jaw-D* and elevated in *miR319a*¹²⁹. Several lines of evidence demonstrate that *TCP4* is a key target of *miR319a*. First, the *tcp4*^{soj6} mutant, which contains a mutation in the *TCP4* miRNA-binding site complementary to the *miR319a*¹²⁹ mutation, suppresses the flower phenotype of *miR319a*¹²⁹. Second, expression of wild-type *TCP4* in petals and stamens (i.e., AP3:*TCP4*) has no effect on flower development; by contrast, a miRNA-resistant version of *TCP4*, when expressed in petals and stamens (i.e., pAP3:m*TCP4*) causes these organs not to develop. Surprisingly, when AP3:*TCP4* is present in a *miR319a*¹²⁹ background, petal and stamen development is severely disrupted, suggesting that proper regulation by *miR319a* of *TCP4* is critical in these floral organs.

DRNL | flower development | forward genetics | microRNA | stamen

MicroRNAs (miRNAs) are 21–24 nucleotide regulatory RNAs that function in diverse aspects of plant biology (1) such as biotic and abiotic stress responses (2), metabolism (3), hormone signaling (4), transcription (5), development (6), and the regulation of miRNA machinery itself (7, 8). Most studies of plant miRNAs focus on alleles that ectopically express the miRNA. In miRNA overexpression lines, the RNA levels of target genes are down-regulated resulting in a phenotype that mimics the loss-of-function phenotypes of miRNA target mutants (9, 10). Loss-of-function alleles in miRNA genes are rare because most often miRNAs are redundantly encoded in plant genomes. Thus, mutation of a single miRNA gene usually does not result in a mutant phenotype. In the few miRNA loss-of-function mutants that are available (5, 6, 11, 12), the RNA levels of target genes are elevated, which is expected if the miRNA can no longer function to degrade target mRNAs.

miR319a was initially characterized based on the dramatic leaf phenotype that results from overexpression of *miR319a*, as in the *jaw-D* allele that results in a jagged and wavy (*jaw*) leaf-phenotype (13). The best-characterized targets of *miR319* are a subset of TCP transcription factors (14). The *TCP* genes are grouped into two subclasses: class I and class II. Class I *TCP* genes, such as *TCP20*, function as positive regulators of cell growth (15) while class II genes like the *Antirrhinum* genes *CIN* (16), *DI-CHOTOMA*, and *CYCLOIDEA* (17, 18) function as negative regulators of cell growth. A subset of class II *TCP* genes contains a *miR319* binding site including *TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24*. In *jaw-D*, due to ectopic expression of *miR319a*, mRNA

levels for the target genes *TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24* are 2- to 34-fold down-regulated in seedlings (13). A role for *TCP* genes in leaf development also comes from analysis of the *cinnata* (*cin*) mutant in *Antirrhinum majus*. *cin* loss-of-function mutants result in uncontrolled cell growth in the leaf margins leading to buckling of the leaf margin (13, 16, 19). In addition, *cin* mutants also exhibit small, slightly curled and pale petals (16). Nath et al. (19) postulate that *CIN* affects leaf development by making cells more sensitive to a growth-arrest signal at the leaf margin.

In *Arabidopsis*, single loss-of-function *tcp* mutants have only very subtle developmental phenotypes. Higher order *tcp* mutants exhibit a phenotype that resembles *jaw-D* suggesting a role for *TCP* genes in leaf development in *Arabidopsis* (20). When an miRNA-sensitive version of *TCP4* is expressed under the broadly expressed cauliflower mosaic virus 35S promoter (i.e., 35S:*TCP4*) the plants fail to exhibit a dramatic leaf or flower phenotype likely due to the presence of *miR319*, which is sufficient to regulate the ectopically expressed *TCP4*. But if the miRNA-binding site of *TCP4* is mutated (i.e., 35S:m*TCP4*) resulting in a form of *TCP4* that is resistant to *miR319*, then ectopic expression results in severe developmental defects including seedling lethality (21). These results underlie the importance of regulating the levels of active *TCP4* in *Arabidopsis* development.

Several lines of evidence also suggest a role for *miR319* in flower development. *jaw-D* mutants exhibit short stamens and reduced male fertility (21). This effect on fertility is postulated to be due to cross-regulation by *miR319* of the MYB genes *MYB33* and *MYB65*. Because *MYB33* and *MYB65* have a poor match to the consensus *miR319*-binding sequence, *MYB33* and *MYB65* are normally secondary targets of *miR319*. Instead, *MYB33* and *MYB65* are regulated more efficiently by *miR159* (21), a miRNA family closely related in sequence to the *miR319* family. While *miR319* has the ability to regulate MYB genes, the mostly non-overlapping expression domains and the low expression level of *miR319* makes *miR319* regulation of the MYB genes biologically insignificant (21).

Based on the overexpression phenotype, *miR319a* was postulated to play a key role in leaf development (13). However, analysis of the expression pattern of *miR319a* indicates that *miR319a* is not expressed in leaves (22). This suggests that *miR319a* might play no role in leaf development, a surprising conclusion in the light of the dramatic leaf phenotype in *jaw-D*. However, it is possible that the other members of the gene family such as *miR319b* and *miR319c* function in leaf development, but whether these two genes are expressed in leaves is not known.

Here, we describe the isolation of a loss-of-function allele of *miR319a*, *miR319a*¹²⁹, which exhibits defects in floral organ morphogenesis. A forward genetic screen was performed in a *dornröschchen-like-2* (*drnl-2*) mutant background to identify modifiers of the

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flower development phenotype; in *drnl-2* mutants filamentous organs develop in whorl 3 in place of stamens and petals are irregularly shaped (23). In *miR319a*¹²⁹ *drnl-2* double mutants, in addition to stamens developing as filamentous structures, second whorl petals also develop as filamentous organs. *miR319a*¹²⁹ single mutants exhibit strongest defects in petal and stamen development. Here, we demonstrate that *miR319a*, *miR319b* and *miR319c* exhibit distinct expression patterns in seedlings and inflorescences, and that *miR319a* is expressed most broadly in developing petals and stamens. We also demonstrate that *TCP4* is a key target of *miR319a* in flowers.

Results

Isolation and Characterization of the *miR319a*¹²⁹ Mutant. The AP2 domain transcription factor DRNL is critical for proper stamen growth in *Arabidopsis* (23). To isolate additional components that function together with *DRNL* in floral organ growth, we performed an enhancer screen in a *drnl-2* background. We identified a strong enhancer (mutant 129) that exhibits dramatic defects in the second whorl of the flower. *drnl-2* single mutants exhibit only minor defects in petal development characterized by irregular length of petals (Fig. 1A); by contrast *drnl-2* 129 double mutants (Fig. 1B and C), exhibit either radialized filaments or dramatically reduced abaxialized petals in whorl 2. In 129 single mutants, developmental defects are present in all four whorls, but the most dramatic defects are in petals and stamens (Fig. 1D). Sepals of 129 single mutants are 18% shorter and often are not tall enough to fully enclose the developing flower (Fig. 1D and J). On an average, mutant petals are 28% shorter and 50% narrower than wild-type petals at anthesis (Fig. 1D and J). The stamens in 129 mutants are shorter (Fig. 1D and J) and anthers are misshapen and lack an organized four-lobed structure (compare Fig. 1E and F). Additionally, stamens are sometimes fused congenitally to the valve of the carpel (0.14 stamen-valve fusions per flower) (Fig. 1H and I). Surprisingly, at least some of these fused stamens are able to produce viable pollen (Fig. 1I).

We cloned the 129 gene by map-based approaches and found that the 129 mutant contains a G to A change at position 12 of the mature *miR319a* (At4g23713) (Figs. 2A and S1). The allele of *miR319a* we isolated (renamed *miR319a*¹²⁹) is the only described loss-of-function allele. All other *miR319a* alleles described to date are overexpression alleles; the best characterized is *jaw-D* (13). The finding that line 129 contains a mutation in *miR319a* is surprising because loss-of-function miRNA mutants are rarely recovered in forward genetic screens due both to the small size of the target and to the redundancy of miRNA gene families. We are aware of only four other loss-of-function miRNA mutants in plants isolated based on mutant phenotype: *miR164c/early extra petals* in *Arabidopsis* (6), *blind* in *Petunia*, *fistulata* in *Antirrhinum* (5), and *tasselseed4* in maize (12). Transformation of the wild-type *miR319a* gene into *miR319a*¹²⁹ rescues the mutant phenotype providing formal proof that the mutation in *miR319a* is responsible for the *miR319a*¹²⁹ phenotype (Fig. 1G).

Computational predictions (24) suggest that this mutation strongly reduces, and likely eliminates, the ability of *miR319a* to recognize its targets. Evidence that *miR319a*¹²⁹ is non-functional comes from experiments where *miR319a*¹²⁹ is ectopically expressed under the 35S promoter; while ectopic expression of wild-type *miR319a* results in a dramatic leaf phenotype (Fig. S2C) (like *jaw-D*), plants that ectopically express *miR319a*¹²⁹ are phenotypically normal (Fig. S2D and E).

***miR319a*, *miR319b*, and *miR319c* Exhibit Largely Non-Overlapping Expression Patterns.** The *miR319a*¹²⁹ mutant exhibits flower-specific phenotypes despite the presence of highly similar and potentially redundant miR319 family members (Fig. 2A). Because the three miRNA products produced by the three miR319 genes are identical (or nearly identical), it is not possible to analyze the expression

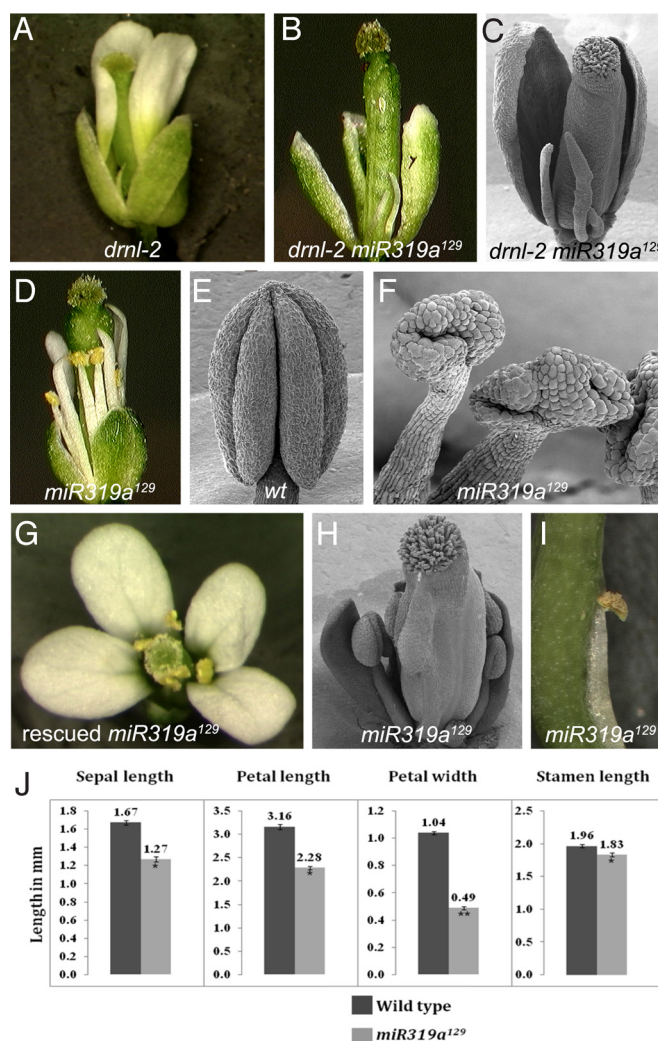


Fig. 1. *miR319a*¹²⁹ mutants exhibit dramatic defects in flower development. (A) *drnl-2* mutant flower. Whorl 3 organs are either missing or develop as small filaments; whorl 2 petals are irregularly shaped. One petal has been removed. (B) *drnl-2 miR319a*¹²⁹ flower. Both second whorl petals and third whorl stamens are converted to filamentous organs. One sepal was manually removed. (C) SEM of a *drnl-2 miR319a*¹²⁹ flower. Two sepals have been removed. (D) *miR319a*¹²⁹ flower. Sepals, petals and stamens are stunted; petals are narrow. (E) Wild-type anther exhibiting characteristic four-lobed structure. (F) Anthers from *miR319a*¹²⁹ flowers are misshapen and lack an organized four-lobed structure. (G) Transformation of a T-DNA that contains a 3.7-kb genomic DNA containing the *miR319a* gene rescues the *miR319a*¹²⁹ flower mutant phenotype. (H) SEM of a *miR319a*¹²⁹ flower showing a stamen congenitally fused to the valve of the carpel. Some perianth organs were manually removed. (I) *miR319a*¹²⁹ flower showing a stamen congenitally fused to the valve of the carpel. The fused anther produces pollen. (J) Floral organ size comparison between wild-type and *miR319a*¹²⁹. Two-sample unequal variance directional *t* test was used to test significance of the difference. All of the numbers were significantly different between wild-type and mutant (*, *P* < 0.05; **, *P* < 0.005). *P* values were 0.013, 0.006, 0.000, and 0.020 for sepal length, petal length, petal width and stamen length respectively. Error bars, standard error of mean (SEM). *n* = 51–121.

pattern of individual gene products using standard techniques such as northern or in situ hybridization. To ascertain if the *miR319a*¹²⁹ phenotype could be attributed to the difference in expression of miR319 family members we analyzed expression of *miR319a*, *miR319b*, and *miR319c* using promoter-GUS fusions. Of the three miR319 genes, *miR319b* exhibits the most restricted expression pattern; GUS activity in *miR319b*:GUS lines is detected only in the

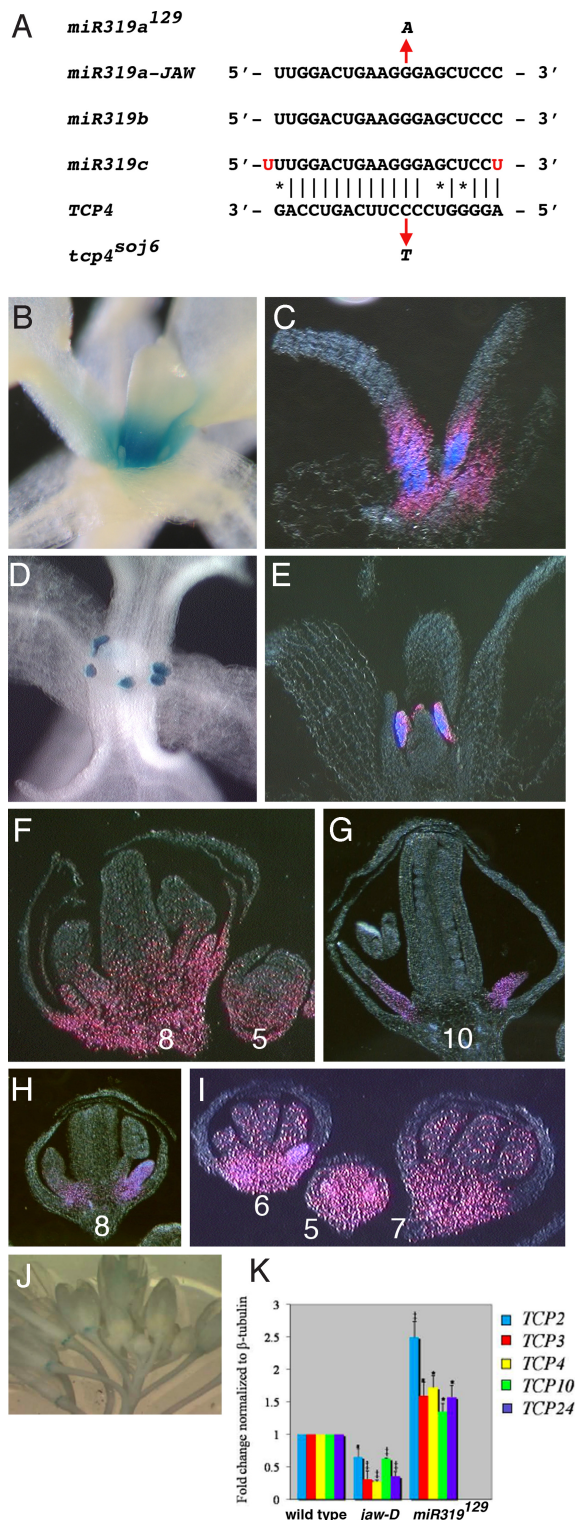


Fig. 2. TCP and miR319 expression. (A) Mature miRNA sequence of three miR319 genes in *Arabidopsis*: *miR319a*, *miR319b*, and *miR319c*. *miR319a* and *miR319b* encode the identical miRNA. The mutation (G to A) in *miR319a*¹²⁹ is indicated by the arrow above the *miR319a* sequence. The *miR319* genes target a subset of TCP transcription factors such as *TCP4*. The *tcp4*^{soj6} mutation (C to T) is indicated by the arrow below the *TCP4* sequence. The *tcp4*^{soj6} and *miR319a*¹²⁹ mutations are complementary. (B) Whole-mount *miR319c*:GUS seedling. The base of young leaf primordia is strongly stained. (C) Section through a *miR319c*:GUS seedling. The base of young leaf primordia is strongly stained. (D) Whole-mount *miR319a*:GUS seedling. Stipules are strongly stained. (E) Section through *miR319a*:GUS seedling. Stipules are strongly stained. (F) Section through

sepal and stamen abscission zones of inflorescences late in flower development (Fig. 2J). By contrast, *miR319a* and *miR319c* are expressed in both seedlings and inflorescences (Fig. 2B–I). In *miR319a*:GUS seedlings, GUS activity is detected at high levels in the stipules, but not in leaf primordia (Fig. 2D and E). In *miR319c*:GUS seedlings, GUS activity is detected at highest levels at the base of leaf primordia and young leaves, but not in the stipules (Fig. 2B and C). The seedling expression of *miR319a* and *miR319c* is completely non-overlapping and, *miR319c*, but not *miR319a*, is expressed in leaves, suggesting that *miR319a* might not play an important role in leaf development.

In *miR319a*:GUS inflorescences, GUS activity is detectable from floral stages 4–11 (25), with highest levels and most persistent expression in developing petals (Fig. 2G–I). In *miR319c*:GUS inflorescences, GUS activity is detectable from floral stages 1–12, with highest levels at the base of all four floral organs and in the pedicel (Fig. 2F). Thus, in the inflorescence, some spatiotemporal overlap of *miR319a* and *miR319c* expression is observed; but only *miR319a* is expressed persistently throughout developing petals. In summary, the expression patterns for the three *miR319* genes in the inflorescence are distinct suggesting that these three genes may have largely unique developmental functions.

TCP Genes Are Up-Regulated in *miR319a*¹²⁹. The best-characterized targets of *miR319* are five TCP genes that have the *miR319* binding sequence: *TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24* (13, 14, 21). In *jaw-D*, RNA levels of these five TCP genes are reduced 2–34-fold in seedlings (13) and 2- to 5-fold in inflorescences (Fig. 2K). Consistent with the prediction that loss-of-function of *miR319a*¹²⁹ would lead to increased expression of the TCP genes, we observe that RNA levels of these five TCP genes are up-regulated between 1.4- and 2.5-fold in *miR319a*¹²⁹ inflorescences (Fig. 2K).

Excess *TCP4* Activity in the Flower Results in Dramatic Floral Defects. To ascertain whether miRNA regulation of *TCP4* is important for flower development, we expressed a *miR319*-resistant form of *TCP4* (*mTCP4*) (13) under the control of the petal- and stamen-specific *AP3* promoter (26, 27). Expression of a wild-type version of *TCP4* under the *AP3* promoter (*AP3*:*TCP4*) has very limited effects on flower development (Fig. 3G, inset) presumably because *miR319* can still regulate *TCP4*. By contrast, strong *AP3*:*mTCP4* lines exhibit a complete absence of petals and stamens (Fig. 3A–C); the flowers are extremely small (Fig. 3C) and the sepals are abnormally fused both to themselves and to the pedicel (Fig. 3A and C). Surprisingly, when *AP3*:*TCP4* is present in a *miR319a*¹²⁹ background (Fig. 3G) petals and sta-

two *miR319c*:GUS flowers. In the stage 5 flower (Right), the pedicel and base of organ primordia are stained. In the stage 8 flower (Left), the petals, stamen filaments, and the base of the carpels and sepals are stained. Flowers staged according to Smyth et al. (25). (G) Section through a stage 10 *miR319a*:GUS flower. The proximal region of the petals is stained. (H) Section through a stage 8 *miR319a*:GUS flower. The petals are strongly stained. (I) Section through three *miR319a*:GUS flowers. In the stage 5 flower (Center) GUS activity is detectable at the base of the sepals and throughout developing second, third, and fourth whorl organs. In the stage 6 (Left) and stage 7 (Right) flowers, GUS activity is detectable at the base of the sepals and carpels, and at high levels throughout developing petals. In stamens, higher levels of GUS activity are detected in the stamen filament primordia compared to anther primordia. (J) Whole-mount *miR319b*:GUS inflorescence. The sepal and stamen abscission zones of late stage flowers are stained. (K) qRT-PCR demonstrates that *TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24* RNA levels increase between 1.4- and 2.5-fold in inflorescences from *miR319*¹²⁹ mutants as compared to wild-type. TCP genes are down-regulated in *jaw-D* inflorescences. Error bars, SEM. All values are significantly different from wild-type. P values for *TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24* are 0.000, 0.028, 0.006, 0.029, and 0.029 respectively for *miR319a*¹²⁹ and 0.026, 0.003, 0.000, 0.001, and 0.003 respectively for *jaw-D*. *, *P* < 0.05, †, *P* < 0.005.

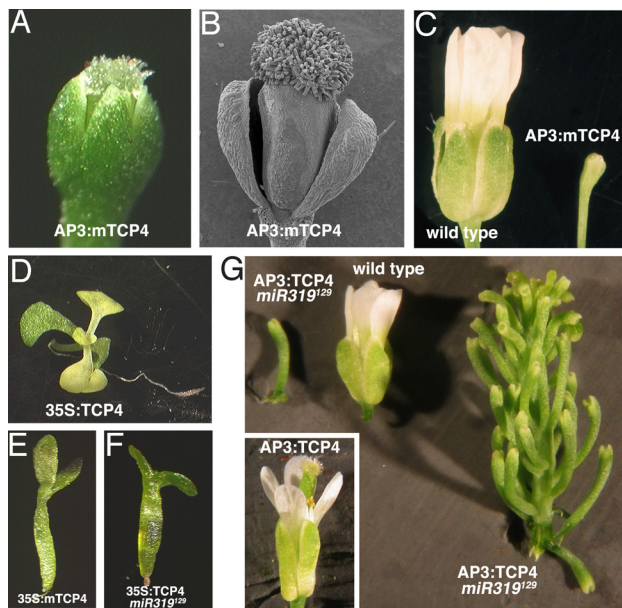


Fig. 3. *miR319a* targets *TCP4*. (A) AP3:mTCP4 flowers consists of only sepals and carpels; petals and stamens are missing. (B) Scanning electron micrograph (SEM) of a AP3:mTCP4 flower. One sepal has been manually removed to allow visualization of the inner whorls. (C) AP3:mTCP4 flower (Right) is dramatically smaller than wild-type flower (Left). (D) 35S:TCP4 seedlings are normal. (E) 35S:mTCP4 seedlings are seedling lethal. (F) 35S:TCP4 *miR319a*¹²⁹ seedlings are seedling lethal and resemble 35S:mTCP4 seedlings. (G) AP3:TCP4 *miR319a*¹²⁹ flowers (Upper Left) and inflorescence (Right). AP3:TCP4 *miR319a*¹²⁹ flowers resemble AP3:mTCP4 flowers (A–C) and are much smaller than wild-type (Center) or AP3:TCP4 flowers (Inset).

mens fail to develop resulting in a flower phenotype identical to AP3:mTCP4.

miR319a regulation of *TCP4* is also important during seedling development. While 35S:TCP4 seedlings develop normally (Fig. 3D), ectopic expression of a *miR319*-resistant form of *TCP4* (35S:mTCP4) results in seedling lethality (Fig. 3E) (13, 28, 29). Interestingly, similar to 35S:mTCP4, 35S:TCP4 leads to seedling lethality in the *miR319a*¹²⁹ background (Fig. 3F). These lines of evidence strongly suggest that *miR319a* is critical for proper regulation of *TCP4* during seedling and flower development.

Suppression of *miR319a*¹²⁹ Phenotype by Compensatory Mutation in *TCP4*. To investigate which of the five TCP genes is most critical for the *miR319a*¹²⁹ phenotype, we made use of the *tcp4*^{soj6} allele which was isolated as a genetic suppressor of the curled leaf phenotype of *jaw-D* (21). *tcp4*^{soj6} contains a mutation in the *miR319* binding site of *TCP4*, at a position complementary to *miR319a*¹²⁹ mutation (Fig. 2A). *tcp4*^{soj6} single mutants are phenotypically normal (Fig. 4A). By contrast, *miR319a*¹²⁹ *tcp4*^{soj6} double mutants exhibit a partial suppression of the second whorl narrow petal phenotype observed in *miR319a*¹²⁹ (Fig. 4C and Fig. S3). The ability of *tcp4*^{soj6} to suppress the *miR319a*¹²⁹ phenotype presumably is due to the restoration of *TCP4* targeting by *miR319a* in the *miR319a*¹²⁹ *tcp4*^{soj6} double mutant. The fact that the petal phenotype of *miR319a*¹²⁹ mutants is not completely suppressed in *miR319a*¹²⁹ *tcp4*^{soj6} double mutants suggests that *miR319a* regulates other genes in petals in addition to *TCP4*, likely *TCP2*, *TCP3*, *TCP10*, and *TCP24*. In contrast to *miR319a*¹²⁹ single mutants, which have a strong flower phenotype, *tcp4*^{soj6} single mutants do not have a flower phenotype, suggesting that regulation of other TCP genes is critical for proper flower development. Despite this caveat, our results suggest that *TCP4* is a key target of *miR319a* in petals. Another line of evidence that *TCP4* is a key regulator in petal development comes from the

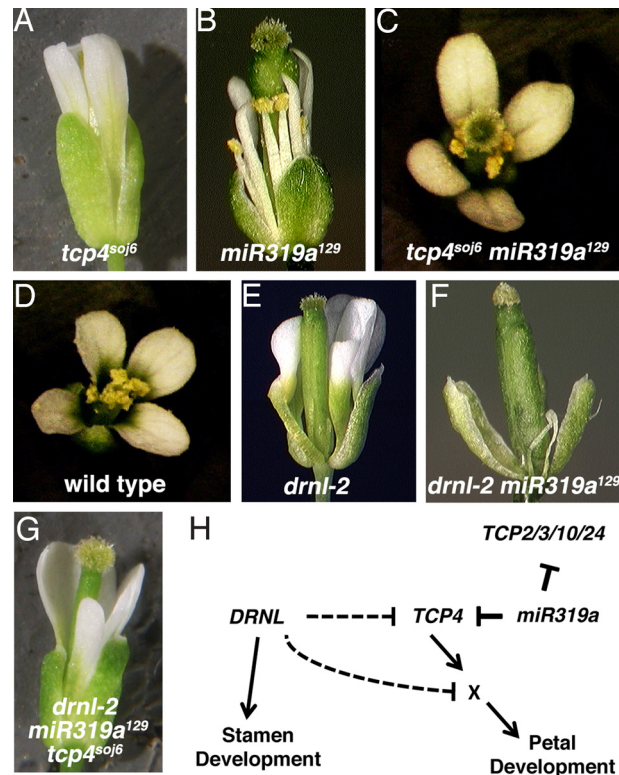


Fig. 4. *tcp4*^{soj6} suppresses the phenotype of both *miR319a*¹²⁹ and *miR319a*¹²⁹ *drnl-2*. (A) *tcp4*^{soj6} flowers have a wild-type phenotype. (B) *miR319a*¹²⁹ flowers have several defects including narrow petals and short stamens. (C) The petals of *tcp4*^{soj6} *miR319a*¹²⁹ double mutants are taller and more lobed than *miR319a*¹²⁹ single mutants. (D) Wild-type flower. (E) *drnl-2* flowers exhibit strongest phenotypic effects in stamens while petals are only mildly affected. (F) *drnl-2* *miR319a*¹²⁹ flowers have dramatically reduced and filamentous petals and stamens. (G) Compared to the *drnl-2* *miR319a*¹²⁹ double mutant (F), the *drnl-2* *miR319a*¹²⁹ *tcp4*^{soj6} triple mutant develops petals in whorl 2. (H) A model for petal development mediated by *DRNL*, *miR319a*, and *TCP4*. *drnl-2* single mutants exhibit only minor defects in petal development, whereas *miR319a*¹²⁹ mutants have narrow petals that are half as wide as wild-type petals. *miR319a*¹²⁹ *drnl-2* double mutants exhibit a very dramatic defect in petal development characterized by filamentous petal formation. The petal defect in the double mutant is rescued by *tcp4*^{soj6} suggesting that the phenotypic enhancement in *drnl-2* is mediated through *TCP4*. Thus, *TCP4* (or a *TCP4* target) is a critical convergence point in the petal development pathway controlled by both *DRNL* and *miR319a*. Although proper regulation of *TCP4* is essential for petal development in a *drnl-2* background, *tcp4*^{soj6} cannot rescue the filamentous stamen phenotype in whorl 3 of *drnl-2* *miR319a*¹²⁹ double mutants indicating the phenotypic defects in whorl 3 of *drnl-2* are not mediated by *TCP4*.

analysis of *drnl-2* *miR319a*¹²⁹ *tcp4*^{soj6} triple mutants (Fig. 4G) in which the filamentous petal defects present in whorl 2 of *drnl-2* *miR319a*¹²⁹ double mutants are rescued (compare Fig. 4F and G). Analysis of expression of the TCP genes via qRT-PCR in *drnl-2* mutant inflorescences indicates that *TCP4* RNA levels are not elevated (Fig. S4). Taken together, these results suggest that although *miR319a* acts directly on *TCP4*, *DRNL* likely functions on the petal development pathway downstream of *TCP4* (Fig. 4H).

Discussion

Previous studies that focused on overexpression alleles of *miR319a* such as *jaw-D*, suggested that *miR319a* played a key role in leaf development (13). Here, we demonstrate that a loss-of-function allele of *miR319a* results in phenotypic defects in flowers, but not leaves, demonstrating that *miR319a* is critical for proper flower development. In *miR319a*¹²⁹ mutants, the most dramatic defects are

*drnl-2 mir319a*¹²⁹ double mutant resulting in second whorl organs that develop as small filamentous organs. In both *drnl-2* and *mir319a*¹²⁹ single mutants, organs clearly recognizable as petals develop, but these organs are not completely wild-type. Thus, *DRNL* and *miR319a* function redundantly with regard to petal growth. The fact that *tcp4*^{soj6} can rescue the petal defects of *drnl-2 mir319a*¹²⁹ double mutants (i.e., the *drnl-2 mir319a*¹²⁹ double mutant lacks petals, but petals are restored in the *drnl-2 mir319a*¹²⁹ *tcp4*^{soj6} triple mutants) suggests that the proper levels of active *TCP4* are critical for proper petal development. *miR319a* directly targets *TCP4* mRNA for degradation, but *DRNL* does not regulate *TCP4* at the level of RNA accumulation (Fig. S4). Most likely, *DRNL* functions downstream of *TCP4*, although we have not ruled out the possibility that *DRNL* functions post-transcriptionally, for example, by altering the level or activity of the *TCP4* protein.

Materials and Methods

Plant Growth Conditions. Plants were grown under continuous light conditions in a 2:1:1 mixture of Promix:perlite:vermiculite at 23 °C. Transgenic plants were selected on Murashige and Skoog medium containing 50 µg/mL kanamycin sulfate or 50 µg/mL BASTA.

***drnl-2* Modifier Screen.** *drnl-2* homozygous seeds were mutagenized with 0.25% ethyl-methane-sulfonate (EMS). Because of the male fertility defects in *drnl-2*, it is difficult to generate a large number of homozygous seeds. Thus, we mutagenized a limited number of *drnl-2* seeds and isolated seeds from 55 M1 plants, and 750 M2 plants were screened. After initial isolation, the 129 mutant was backcrossed to wild-type Ler, and in the F2 of the backcross, plants with the following phenotypes segregated: 179 wild-type: 63 *drnl-2*: 66 *mir319a*¹²⁹: 22 *drnl-2 mir319a*¹²⁹, a ratio consistent with segregation of two unlinked nuclear genes in a 9:3:3:1 ratio.

Map-Based Cloning of *mir319a*¹²⁹. *drnl-2 mir319a*¹²⁹ mutants were crossed to Columbia and DNA samples were prepared from individual mutant F2 plants (33). Two independent pools of 17 and 21 samples were subjected to bulk-segregant analysis (34). 411 independent *mir319a*¹²⁹ homozygous mutants were used to perform fine mapping using SSLP, CAPS, and dCAPS markers (Table S1) designed using information available on TAIR ([\[dopsis.org/\]\(http://dopsis.org/\)\) or reported by CERION \(34\). Gene annotations from TAIR were used to identify candidate genes.](http://www.arabi-</p>
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Scanning Electron Microscopy. Samples were fixed and visualized as described in ref. 23.

Floral Organ Size Measurement. Floral organs from anthesis stage flowers were used for scoring. The floral organs were flattened on sticky side of a cellophane tape and photographed. A photograph of a ruler was taken using same magnification was used as a calibration tool. SPOT-Advanced software (Diagnostic Instruments, Inc.) was used to calculate the length of floral organs.

Real-Time qRT-PCR Experiments. One to two micrograms total RNA extracted using the RNeasy mini kit (Qiagen) was used for random-hexamer primed cDNA synthesis with SuperScript III 1st Strand Synthesis SuperMix (Invitrogen catalog no. 18080-400). The resulting cDNA was subjected to relative quantitative PCR in presence of iQ SYBR Green Supermix (Bio-Rad, catalog no. 170-8882) in a Bio-Rad iCycler or SYBR Premix Ex Taq II (Perfect Real Time) (TaKaRa, cat. #RR081A) in an ABI 7500 Real-Time PCR System. For each reported result at least three independent biological samples were subjected to minimum of three technical replicates. The results were normalized to β -tubulin (*TUB2/TUB3*). The primers used are reported in Table S2 (13).

Plasmid Constructs and Transgenic Lines. The constructs used are listed in Table S3. The two complementation constructs containing 1.2- or 1.6-kb promoter sequence were both able to complement *mir319a*¹²⁹ phenotype. For the promoter:GUS constructs we used 1.2-, 2.9-, and 2.6-kb regions 5' to the predicted transcription start site for *miR319a*, *miR319b*, and *miR319c* respectively. Forty-five of 46 AP3:mTCP4 lines generated exhibit small flowers with reduced petals and stamens. All 21 independent AP3:TCP4 transgenic lines in *mir319a*¹²⁹ background exhibit phenotypes similar to those described for AP3:mTCP4. The expression pattern we report here was observed in 12/14, 9/12, and 6/10 independent lines generated for the *miR319a*:GUS, *miR319b*:GUS, and *miR319c*:GUS constructs respectively.

GUS Staining. Promoter:GUS fusion lines were stained using a standard protocol for 24–48 h (35).

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