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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:mTCP4) 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 *ja*gged and *w*avy (*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 *CINCINNATA* (*CIN*) (16), *DICHOTOMA*, 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 *cincinnata* (*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:mTCP4) 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 *dornröschen-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. 1*A*); by contrast *drnl-2 129* double mutants (Fig. 1 *B* 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. 1*D*). Sepals of *129* single mutants are 18% shorter and often are not tall enough to fully enclose the developing flower (Fig. 1 *D* and *J*). On an average, mutant petals are 28% shorter and 50% narrower than wild-type petals at anthesis (Fig. 1 *D* and *J*). The stamens in *129* mutants are shorter (Fig. 1 *D* and *J*) and anthers are misshapen and lack an organized four-lobed structure (compare Fig. 1 *E* and *F*). Additionally, stamens are sometimes fused congenitally to the valve of the carpel (0.14 stamen-valve fusions per flower) (Fig. 1 *H* and *I*). Surprisingly, at least some of these fused stamens are able to produce viable pollen (Fig. 1*I*).

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. 2*A* 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. 1*G*).

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. S2*C*) (like *jaw-D*), plants that ectopically express *miR319a*^{129} are phenotypically normal (Fig. S2 *D* 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. 2*A*). Because the three miRNA products produced by the three miR319 genes are identical (or nearly identical), it is not possible to analyze the expression 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

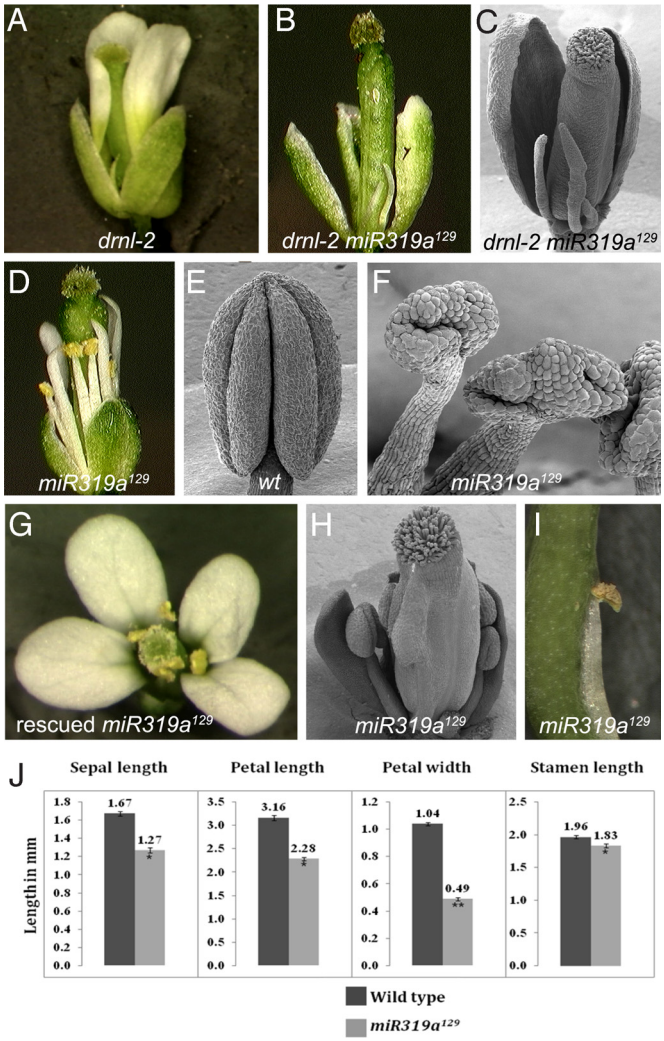

Fig. 1. *miR319a*^{129} mutants exhibit dramatic defects in flower development. (*A*) *drnl-2* mutant flower. Whorl 3 organs are either missing or develop as small filaments; whorl 2 petals are irregularly shaped. One petal has been removed. (*B*) *drnl-2 miR319a*^{129} flower. Both second whorl petals 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). $n = 51–121$.

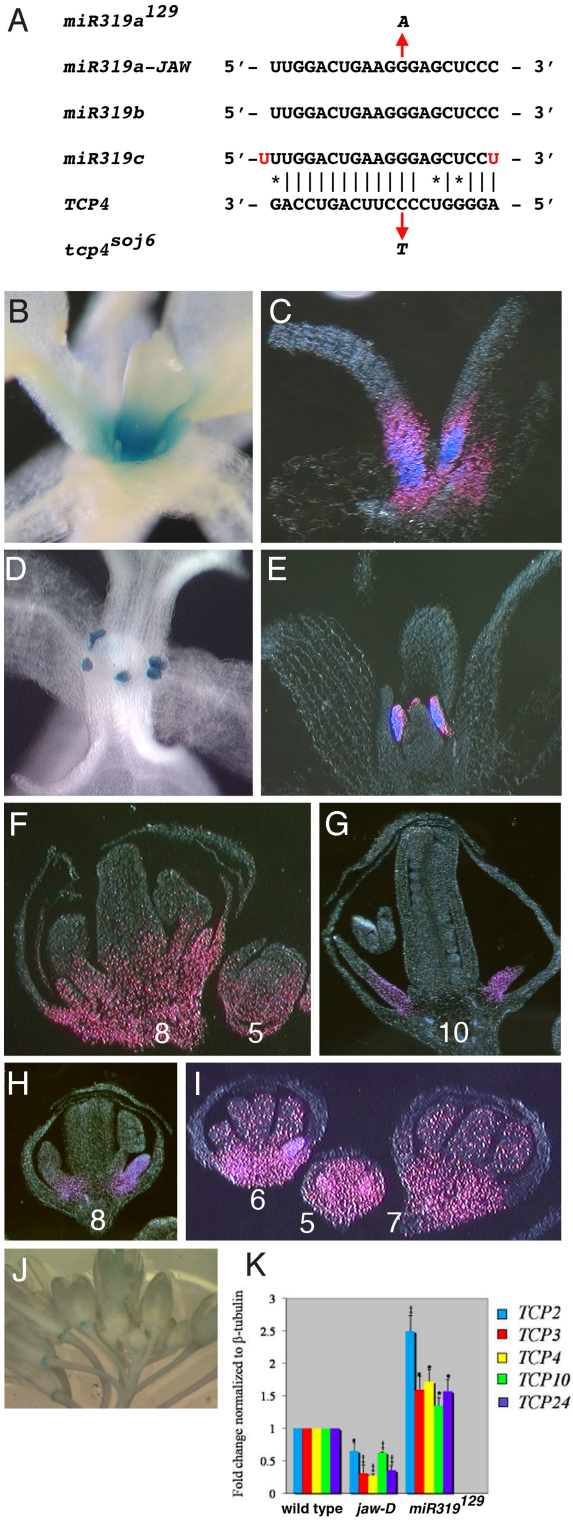

Fig. 2. TCP and miR319 expression. (*A*) Mature miRNA sequence of three miR319 genes in *Arabidopsis*: *miR319a*, *miR319b*, and *miR319c*. *miR319a* and *miR319b* encode the identical miRNA. The mutation (G to A) in *miR319a*^{129} is indicated by the arrow above the *miR319a* sequence. The *miR319* 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 priordia 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 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*. ⋆, $P < 0.05$, ‡, $P < 0.005$.

sepal and stamen abscission zones of inflorescences late in flower development (Fig. 2*J*). By contrast, *miR319a* and *miR319c* are expressed in both seedlings and inflorescences (Fig. 2 *B–I*). In miR319a:GUS seedlings, GUS activity is detected at high levels in the stipules, but not in leaf primordia (Fig. 2 *D* 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. 2 *B* 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. 2 *G–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. 2*F*). 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. 2*K*). 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. 2*K*).

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. 3*G*, inset) presumably because miR319 can still regulate *TCP4*. By contrast, strong AP3:mTCP4 lines exhibit a complete absence of petals and stamens (Fig. 3 *A–C*); the flowers are extremely small (Fig. 3*C*) and the sepals are abnormally fused both to themselves and to the pedicel (Fig. 3 *A* and *C*). Surprisingly, when AP3:TCP4 is present in a *miR319a*^{129} background (Fig. 3*G*) petals and sta-

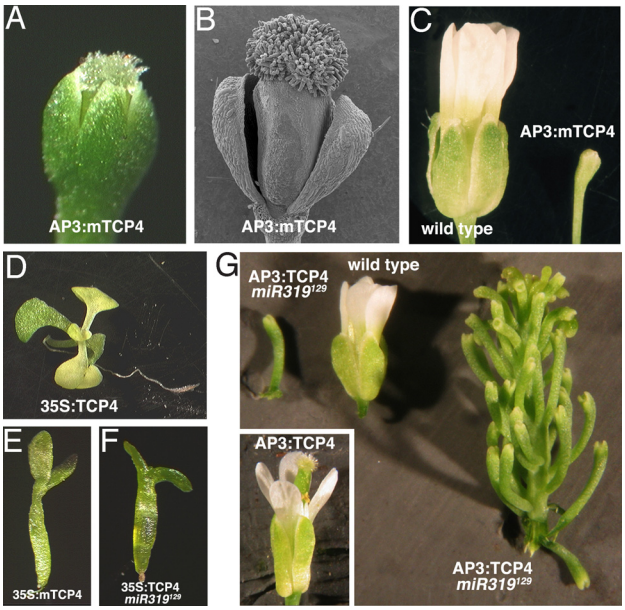

Fig. 3. *miR319a* targets *TCP4*. (*A*) AP3:mTCP4 flowers consists of only sepals and carpels; petals and stamens are missing. (*B*) Scanning electron micrograph (SEM) of a AP3:mTCP4 flower. One sepal has been manually removed to allow visualization of the inner whorls. (*C*) AP3:mTCP4 flower (*Right*) 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*).

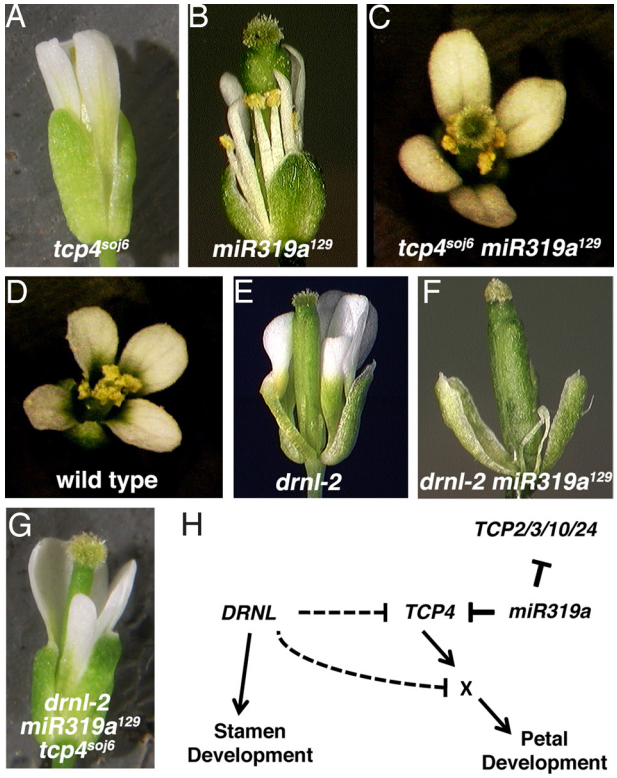

Fig. 4. *tcp4*^{soj6} suppresses the phenotype of both *miR319a*^{129} and *miR319a*^{129} *drnl-2*. (*A*) *tcp4*^{soj6} flowers have a wild-type phenotype. (*B*) *miR319a*^{129} flowers have several defects including narrow petals and short stamens. (*C*) The petals of *tcp4*^{soj6} *miR319a*^{129} 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*.

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. 3*D*), ectopic expression of a miR319-resistant form of *TCP4* (35S:mTCP4) results in seedling lethality (Fig. 3*E*) (13, 28, 29). Interestingly, similar to 35S:mTCP4, 35S:TCP4 leads to seedling lethality in the *miR319a*^{129} background (Fig. 3*F*). 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. 2*A*). *tcp4*^{soj6} single mutants are phenotypically normal (Fig. 4*A*). By contrast, *miR319a*^{129} *tcp4*^{soj6} double mutants exhibit a partial suppression of the second whorl narrow petal phenotype observed in *miR319a*^{129} (Fig. 4*C* 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 analysis of *drnl-2 miR319a*^{129} *tcp4*^{soj6} triple mutants (Fig. 4*G*) in which the filamentous petal defects present in whorl 2 of *drnl-2 miR319a*^{129} double mutants are rescued (compare Fig. 4 *F* 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. 4*H*).

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

in petals and stamens. The isolation of the loss-of-function *miR319a*^{129} allele in a forward genetic screen is surprising since *miR319* is redundantly encoded in the Arabidopsis genome; thus, loss of a single *miR319* gene would not be expected to result in a mutant phenotype because of this redundancy. However, we show that the three miR319 genes exhibit largely non-overlapping spatial expression patterns in seedlings and inflorescences, and that *miR319a* is the only one of the three *miR319* genes that is broadly and persistently expressed in developing petals, likely explaining why we see the strongest phenotypic defects in these organs in *miR319a*^{129} mutants. Thus, the three members of the miR319 family exhibit distinct expression patterns suggesting that they possess unique developmental functions. The precise developmental roles of *miR319b* and *miR319c* will be clarified when loss-of-function alleles become available.

It is well-established that the key targets of *miR319* are a subset of TCP transcription factors, specifically *TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24* (13, 21). In *jaw-D*, these five TCP genes are dramatically down-regulated in seedlings as a result of targeting of TCP transcripts for degradation by *miR319a*. A similar, although less dramatic, reduction in TCP expression is observed in *jaw-D* inflorescences (Fig. 2*K*). In the loss-of-function *miR319a*^{129} mutant, we see an increase in *TCP* RNA levels in inflorescences (Fig. 2*K*). The increase (1.4- to 2.5-fold) that we observe is not as dramatic as the decrease observed in *jaw-D*; however, this is not surprising since *miR319a* is expressed in only a subset of cells in inflorescences (Fig. 2 *G–I*), so in essence we are observing an increase in *TCP* targets in a small number of cells of the inflorescence that express *miR319a*.

***miR319* and Leaf Development.** The results of this study raise the question as to whether *miR319a* has a function in normal leaf development. Our analysis of the expression pattern of *miR319a* indicates that the *miR319a* promoter is not active in developing leaves (Fig. 2 *D* and *E*) (22). By contrast, the *miR319c* promoter is active in the proximal region of developing leaves (Fig. 2 *B* and *C*). This suggests that *miR319c*, but not *miR319a* might function in leaf development. Since *miR319a* and *miR319c* produce very similar miRNA products, the overexpression leaf phenotype of *jaw-D* might result from *miR319a* regulating normal *miR319c* targets in the leaf.

Although the *TCP2*, *TCP3*, *TCP4*, and *TCP24* promoters are active throughout leaves (30), *TCP4* RNA is detected only in the distal end of the leaf (13). *miR319c* is expressed in the proximal region of the leaf and likely targets TCP RNAs for degradation. Similar expression patterns are observed in tomato leaves, where there is an opposing and overlapping gradient of *LANCEOLATE* (*LA*) RNA (a *TCP3*/*TCP4* ortholog) and a *miR319* family member (28). The *LA* transcript accumulates at the distal end of the leaf whereas tomato *miR319* is expressed at high levels in the proximal leaf region. It is hypothesized that the relative levels of *miR319* and *LA* in different parts of the tomato leaf regulate the developmental fate of the leaf (28). In the proximal region of the leaf where the *miR319* level is high and the *LA* level is low, organ initiation is promoted, while in the distal region of the leaf, where the miR319 level is low and *LA* levels are high, organ differentiation is favored (28). Since there is no overlap in expression of miR319 family members and *TCP4* in the distal part of the leaf and *miR319c* is expressed strongly at the base of the leaves (13, 28), it is tempting to speculate that a similar proximal-distal specification circuit occurs in *Arabidopsis* as well.

Does TCP4 Function Non-Cell-Autonomously? miRNA-resistant forms of *TCP4* (*mTCP4*) have dramatic effects on plant development. Expression of *mTCP4* under the control of the broadly expressed CaMV 35S promoter results in seedling lethality (Fig. 3*E*) (13). Previous studies suggest that *TCP4* is a negative regulator of cell proliferation (16, 19). Thus, when constitutively active (i.e., miRNA-resistant) forms of *TCP4* are expressed in plants, organ development and differentiation are suppressed. This activity of *TCP4* explains, in part, the phenotype of plants that express mTCP4 under the control of the petal and stamen-specific *AP3* promoter (Fig. 3 *A–C*). In pAP3:mTCP4 plants, petals and stamens do not develop (Fig. 3*B*), likely as a result of constitutively active forms of *TCP4* being expressed during very early stages of flower development in petal and stamen primordia. Surprisingly, the phenotype of AP3:mTCP4 flowers is not confined to petals and stamens. AP3:mTCP4 flowers are much smaller than wild-type and the sepals are abnormally fused both to each other and to the pedicel (Fig. 3 *A–C*). These phenotypes outside of the petals and stamens suggest that *mTCP4* may function non-cell-autonomously; that is, that expression of *mTCP4* in the petals and stamens is altering the development of surrounding cells and tissues. It is unlikely that these effects are due to leakiness of the *AP3* promoter, and subsequent low-level expression of *mTCP4* in cells outside the petals and stamens. The best evidence for this comes from analysis of lines that ectopically express diptheria toxin (DT-A) under the control of the *AP3* promoter (31). In the AP3:DT-A transgenic lines, petals and stamens fail to develop, but other floral organs (sepals and carpels) develop normally and the flowers are of normal size. Thus, the phenotype of AP3:mTCP4 likely is due to the fact that *mTCP4* functions non-cell-autonomously in the flower, similar to what has been observed with other TCP genes such as *TCP14* (32). However, the data that we have assembled at present falls short of formal proof that *TCP4* functions non-cell-autonomously. It is also possible that one or more downstream targets of *TCP4* functions non-cell-autonomously or that expression of excess *TCP4* in whorl 2 and whorl 3 during very early stages of flower development grossly alters cell growth and division leading to a dramatic change in surrounding floral structures.

Controlling the Level of Active *TCP4* Is Critical for Proper Petal and Stamen Development. In *miR319a*^{129}, the transcript levels for all five TCP targets are up-regulated. To identify which of the TCP targets mediate the defects observed in *miR319a*^{129}, we used two different approaches. First we expressed a miRNA non-regulatable version of *TCP4* in petals and stamens. This resulted in either severe reduction of petal and stamen formation or a complete failure of petal and stamen development (Fig. 3 *A* and *B*). Interestingly, expression of wild-type *TCP4* using the petal- and stamen-specific *AP3* promoter, in the *miR319a*^{129} background also led to a failure of petal and stamen development (Fig. 3*G*). This indicates that a failure to down-regulate *TCP4* in petals and stamens leads to severe developmental defects. Additionally, a similar experiment using the 35S promoter suggests that excess *TCP4* can only be tolerated in presence of functional *miR319a* during early seedling development (Fig. 3*F*).

A more convincing line of evidence that *TCP4* is a key target of *miR319a* comes from the analysis of double mutants between *miR319a* and *tcp4*^{soj6}. The mutation in *tcp4*^{soj6} is complimentary to the mutation in *miR319a*^{129} (Fig. 2*A*). In the *tcp4*^{soj6} *miR319a*^{129} double mutant, the prediction is that the mutant *miR319a* should be able to target the mutant *TCP4* mRNA for degradation. We observe a partial rescue of the flower phenotype in the *tcp4*^{soj6} *miR319a*^{129} double mutant (Fig. 4C and Fig. S3) indicating that *TCP4* mediates many aspects of the phenotype observed in *miR319*^{129}. But the fact that a wild-type petal phenotype is not restored in *miR319a*^{129} *tcp4*^{soj6} double mutants suggests that *miR319a* regulates other genes in petals in addition to *TCP4*; likely some combination of *TCP2*, *TCP3*, *TCP10*, and *TCP24*.

A Model for Petal Development Mediated by *DRNL*, *miR319a*, and *TCP4*. In this study, we have uncovered a role for *miR319a* in flower development, specifically in petal and stamen development (Fig. 4*H*). In *miR319a*^{129} single mutants, the petals are narrow and the stamens have defects in filament length and anther morphology. The *miR319a*^{129} petal phenotype is dramatically enhanced in the

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 L-*er*, 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 (http://www.arabidopsis.org/) or reported by CEREON (34). Gene annotations from TAIR were used to identify candidate genes.

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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