Roles of miR319 and TCP Transcription Factors in Leaf Development[1][OPEN]

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Sophisticated regulation of gene expression, including microRNAs (miRNAs) and their target genes, is required for leaf differentiation, growth, and senescence. The impact of miR319 and its target *TEOSINTE BRANCHED1*, *CYCLOIDEA*, and *PROLIFERATING CELL NUCLEAR ANTIGEN BINDING FACTOR* (*TCP*) genes on leaf development has been extensively investigated, but the redundancies of these gene families often interfere with the evaluation of their function and regulation in the developmental context. Here, we present the genetic evidence of the involvement of the *MIR319* and *TCP* gene families in Arabidopsis (*Arabidopsis thaliana*) leaf development. Single mutations in *MIR319A* and *MIR319B* genes moderately inhibited the formation of leaf serrations, whereas double mutations increased the extent of this inhibition and resulted in the formation of smooth leaves. Mutations in *MIR319* and gain-of-function mutations in the *TCP4* gene conferred resistance against miR319 and impaired the cotyledon boundary and leaf serration formation. These mutations functionally associated with *CUP-SHAPED COTYLEDON* genes, which regulate the cotyledon boundary and leaf serration formation. In contrast, loss-of-function mutations in miR319-targeted and nontargeted *TCP* genes cooperatively induced the formation of serrated leaves in addition to changes in the levels of their downstream gene transcript. Taken together, these findings demonstrate that the *MIR319* and *TCP* gene families underlie robust and multilayer control of leaf development. This study also provides a framework toward future researches on redundant miRNAs and transcription factors in Arabidopsis and crop plants.

Leaves are differentiated at the flank of shoot meristems, later forming their shapes and senescing at the final stage of their development (Bar and Ori, 2014; Ichihashi and Tsukaya, 2015). The integration of diverse developmental programs during leaf development requires a complex and robust network of gene regulation. This regulation requires the sophisticated balance between microRNAs (miRNAs) and their target genes (Rodriguez et al., 2016; Fouracre and Poethig, 2016).

miR319 and its target genes, *TEOSINTE BRANCHED1*, *CYCLOIDEA*, and *PROLIFERATING CELL NUCLEAR ANTIGEN BINDING FACTOR* (*TCP*), play pivotal roles in leaf development. Among 24 *TCP* genes in Arabidopsis (*Arabidopsis thaliana*), the phylogenetically related *CINCINNATA*-like *TCP* subfamily consists of five miR319-targeted genes, namely, *TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24*, as well as three nontargeted genes (Supplemental Fig. S1; Nath et al., 2003; Palatnik et al., 2003; Lopez et al., 2015; Nicolas and Cubas, 2016; named here as *TCP* for simplicity). Arabidopsis knockout mutants for three or more *TCP* genes exhibit serrated and wavy leaves (Koyama et al., 2010), indicating that these *TCP* genes redundantly regulate the margin and surface of leaves. Moreover, chimeric *TCP* repressor genes, in which the *TCP* genes are fused with the strong repression domain, generate serrated and wavy leaves in transgenic Arabidopsis, torenia, chrysanthemum, cyclamen, rose, and morning glory plants, suggesting the conserved role of these *TCP* genes in leaf development (Hiratsu et al., 2003; Koyama et al., 2007, 2011; Narumi et al., 2011; Gion et al., 2011; Tanaka et al., 2011; Ono et al., 2012; Li and Zachgo 2013; Sasaki et al., 2016).

These actions of TCP transcription factors are largely dependent on the negative regulation of the *CUP-SHAPED COTYLEDON1* (*CUC1*), *CUC2*, and *CUC3* genes (Koyama et al., 2007, 2010; Rubio-Somoza et al., 2014). *CUC* genes are active in the boundary of two cotyledons and are required for the formation of the sinus (Aida et al., 1997; Aida and Tasaka, 2006; Hibara et al., 2006). *CUC2* and *CUC3* genes are also active in the sinuses of the leaf margin and are required for leaf

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serration (Nikovics et al., 2006; Hasson et al., 2011; Maugarny et al., 2016). The TCP transcription factors negatively regulate the expression of *CUC* genes via genes downstream of the TCPs (Koyama et al., 2007, 2010). Moreover, TCP4 directly interacts with CUC2 and CUC3 and inhibits their activities at the post-translational level (Rubio-Somoza et al., 2014).

miR319 partially but significantly controls the activity of *TCP* genes. The nearly complementary nucleotide sequences of *TCP* mRNAs and miR319 underlie the specific mechanism of gene regulation (Palatnik et al., 2003, 2007; Ori et al., 2007; Shleizer-Burko et al., 2011). A single-nucleotide mutation of miR319 reduces its ability to target the five *TCP* mRNAs (Nag et al., 2009; Supplemental Fig. S2). Mutations in *TCP* genes, which are resistant against miR319 (*mTCP*), induce gain-of-function effects and induce impairments in the shoot meristem, cotyledon boundary, leaf margin, and size (Palatnik et al., 2003, 2007; Koyama et al., 2007; Efroni et al., 2008; Sarvepalli and Nath, 2011; Schommer et al., 2014). Conversely, ectopic expression of *MIR319* in Arabidopsis decreases the levels of the five *TCP* transcripts and results in the formation of jagged and wavy leaves (Palatnik et al., 2003, 2007; Koyama et al., 2007; Efroni et al., 2008). miR319 is encoded by three genes in the Arabidopsis genome, and this apparent redundancy may mask the leaf irregularities observed with a loss-of-function allele of miR319 (Nag et al., 2009). Overexpression of an inhibitory RNA against miR319 (target mimicry; MIM319) implies a dominant-negative effect of miR319 (Todesco et al., 2010; Rubio-Somoza and Weigel, 2013); however, the predominant mechanism through which *MIR319* genes act during leaf development remains unclear.

Because of the redundancies of *TCP* and *MIR319* genes, the previous studies on these genes had been often dependent on data derived from dominant-negative and active methods (Nicolas and Cubas, 2016). In contrast, generating multiple mutants that include novel alleles for the *MIR319* and *TCP* genes, we here demonstrate that these mutants changed the leaf and cotyledon morphology more gradually than previously thought. In this study, we obtained genetic evidence that miR319 acts in the formation of the cotyledon boundary and leaf serrations in association with *CUC* genes. We also generated sextuple *tcp* mutants and transgenic plants, in which the eight *TCP* genes were almost suppressed, in order to delineate the role of the highly redundant *TCP* genes in leaf development. Our results provide a framework toward future researches on miRNAs and transcription factors that redundantly regulate important traits such as development, physiology, and reproduction in Arabidopsis and crop plants.

RESULTS

Characterization of Loss-of-Function Alleles for *MIR319A* and *MIR319B*

Since the accumulation of miRNAs is usually associated with the transcription of precursor genes, we first examined which of the precursor genes *MIR319A*, *MIR319B*, and *MIR319C* were active during leaf development in Arabidopsis. The results of RT-PCR analysis demonstrated that the transcript level of *MIR319A* was largely constant during plant development, whereas those of *MIR319B* and *MIR319C* differentially increased in vegetative and reproductive organs, respectively (Supplemental Fig. S3). We expected that *MIR319A* and *MIR319B* would contribute to the regulation of leaf development.

Next, we generated loss-of-function mutants of *MIR319A* and *MIR319B* to investigate the functions of miR319 during leaf development. *mir319a*^{123}, which carries a nucleotide replacement in the complementary sequence of *MIR319A* to the *TCP* genes (Nag et al., 2009; Supplemental Fig. S2), was crossed with Colombia-0 to standardize the genetic background (hereafter, the resultant mutant is referred to as *mir319a* to discriminate the Colombia-0 background). *mir319b* was newly identified in this study from the GABI_KAT mutant collection (Rosso et al., 2003), in which the expression of the *MIR319B* gene was lost due to the insertion of T-DNA (Supplemental Fig. S4, A and B). We further investigated whether the loss-of-function allele of *MIR319B* could cause inability to target *TCP3* mRNA. When the *TCP3* gene construct, under the control of the constitutively active cauliflower mosaic virus 35S promoter (Pro-35S), was introduced into wild-type plants, the resultant transgenic plants (*Pro-35S:TCP3*) formed normal leaves due to the activity of miR319 (Koyama et al., 2007; Supplemental Fig. S4, C and D). In contrast, the *TCP3* construct was introduced into *mir319b*, and the resultant *Pro-35S:TCP3 mir319b* exhibited cotyledon fusion and smooth leaves (Supplemental Fig. S4, C–E), as observed in *Pro-35S:mTCP3* plants (Supplemental Fig. S2; Koyama et al., 2007, 2010). This suggested that the ability to target the ectopically induced *TCP3* mRNA was substantially impaired in *mir319b*.

Impaired Formation of Leaf Serrations, Hypocotyl, and Inflorescence in *mir319a/b*

The transgenic plants carrying the *mTCP2*, *mTCP3*, or *mTCP4* gene generate leaves with a smooth margin (Palatnik et al., 2003; Koyama et al., 2007, 2010; Schommer et al., 2014); therefore, we investigated this phenotype of the *mir319*-related mutants. The sixth leaf of the wild type usually generated a serration on each side of the margin, whereas the leaves of *mir319a* and *mir319b* had serrations of reduced size (Fig. 1, A and B). When *mir319a* was crossed with *mir319b*, the resultant *mir319a mir319b* double mutant (*mir319a/b*) exhibited reduced serrations and, as observed in the severely defected cases, in eight out of 19 *mir319a/b* plants analyzed, no serrations were observed (Fig. 1, A and B). These results clarified an additive effect of the *mir319a* and *mir319b* mutations on the formation of leaf serrations.

In addition, *mir319a/b* had a longer hypocotyl than wild type under the light condition (Fig. 1, C and D).

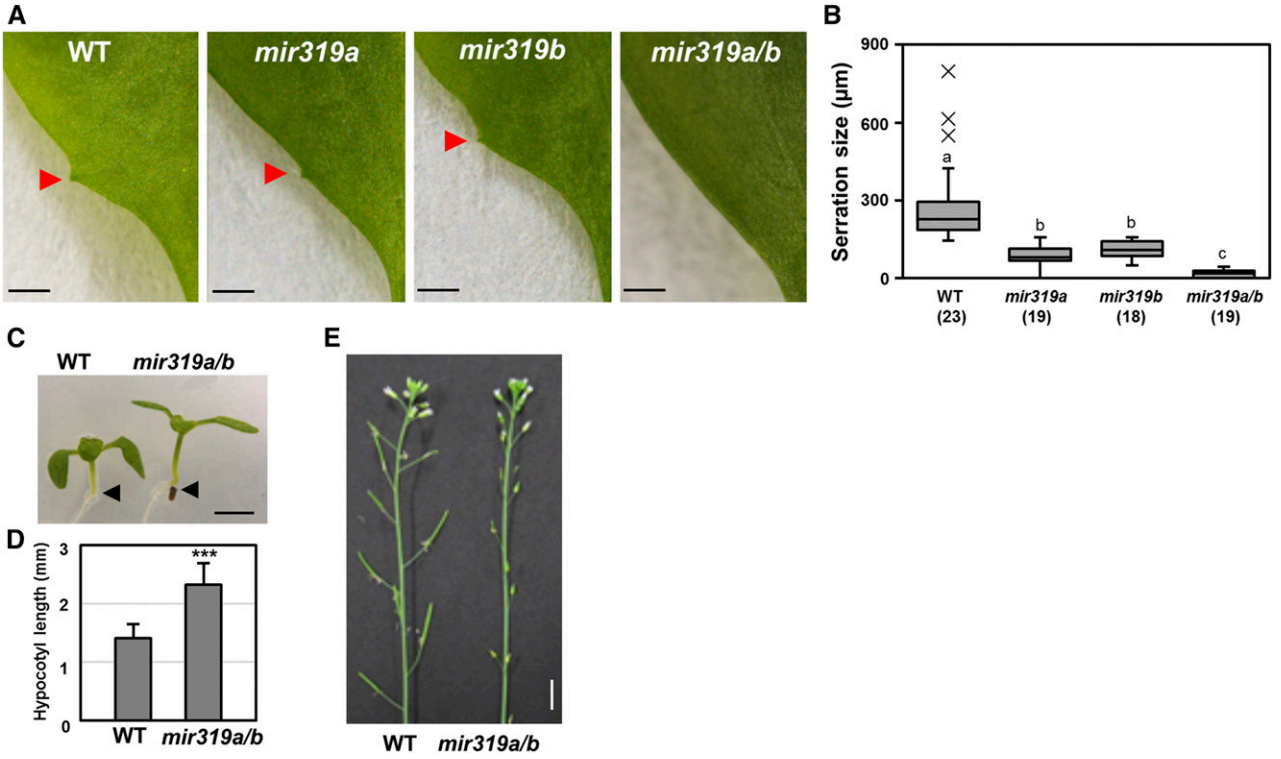

Figure 1. The effects of *mir319a* and *mir319b* mutations on leaf serration formation, fruit bearing, and hypocotyl elongation. A, Photograph showing the sixth leaves of wild-type, *mir319a*, *mir319b*, and *mir319a/b* plants. Triangles indicate the serration at the leaf margin. Bars = 1 mm. B, Quantification of serration size in the sixth leaves of wild-type, *mir319a*, *mir319b*, and *mir319a/b* plants. The different letters and crosses in the box plot indicate a statistically significant difference (Tukey-Kramer method; $P < 0.05$) and outliers, respectively. Numbers in parentheses below the genotypes represent biological replicates. C, Photograph showing the hypocotyls of wild-type and *mir319a/b* seedlings. Triangles indicate the junction of the hypocotyl and root. Bar = 1 mm. D, Quantification of the hypocotyl length of wild-type and *mir319a/b* seedlings. The means were calculated from 20 hypocotyls of 7-d-old seedlings. Error bars and asterisks indicate SD and significant difference (Student *t* test; $***P < 0.001$). E, The primary inflorescences of wild-type and *mir319a/b* plants. Bar = 1 cm

These changes in *mir319a/b* are consistent with the moderate phenotype of transgenic plants overexpressing the *mTCP* genes (Palatnik et al., 2003; Koyama et al., 2007).

After floral organs senesce in the reproductive growth, wild-type plants expand their siliques, which are usually signs of successful pollination and subsequent embryogenesis (Fig. 1E). *mir319a* and *mir319b* were also fertile, whereas *mir319a/b* rarely exhibited silique expansion and less than 10 seeds were produced per plant (Fig. 1E). Similar abnormalities in the *Pro-35S:mTCP3* and *MIM319* plants suggested that the *MIR319A* and *MIR319B* genes functioned in the reproductive organs via the regulation of *TCP* genes (Supplemental Fig. S6; Rubio-Somoza and Weigel, 2013).

Effects of *mir319a* and *mir319b* Mutations on the Expression of *TCP* Genes

The overexpression of *MIR319A* reduces the levels of the miR319-targeted *TCP* genes in leaves (Palatnik et al., 2003, 2007), but effects of the loss-of-function of *MIR319A* and *MIR319B* on the *TCP* transcripts are obscure. We examined the effects of *mir319* mutations on the levels of the *TCP* transcripts. Our RT-PCR analysis demonstrated that transcripts of the five miR319-targeted *TCP* genes increased in *mir319a/b* seedlings in comparison to wild-type seedlings (Fig. 2A). In contrast, several *TCP* transcripts moderately increased in *mir319a*, but none of them increased in *mir319b* (Supplemental Fig. S5), suggesting that each mutation alone might cause mild effects on the levels of the *TCP* transcripts.

mir319 Mutations Enhanced Effects of *cuc* Mutations on Cotyledon Boundary and Leaf Serration

To investigate the effects of the *mir319a/b* mutation on the expression levels of TCP downstream genes, we conducted RT-PCR analysis of wild type and *mir319a/b* seedlings. The transcript levels of *SHORT HYPOCOTYL2/INDOLE-3-ACETIC ACID3* (*SHY2/IAA3*), *SMALL AUXIN UP RNA* (*SAUR*), *miR164A*, and *LIPOXYGENASE2* (*LOX2*), which are direct targets of the TCP3, TCP4, and TCP10 transcription factors (Schommer et al., 2008; Koyama et al., 2010; Danisman et al., 2013), were

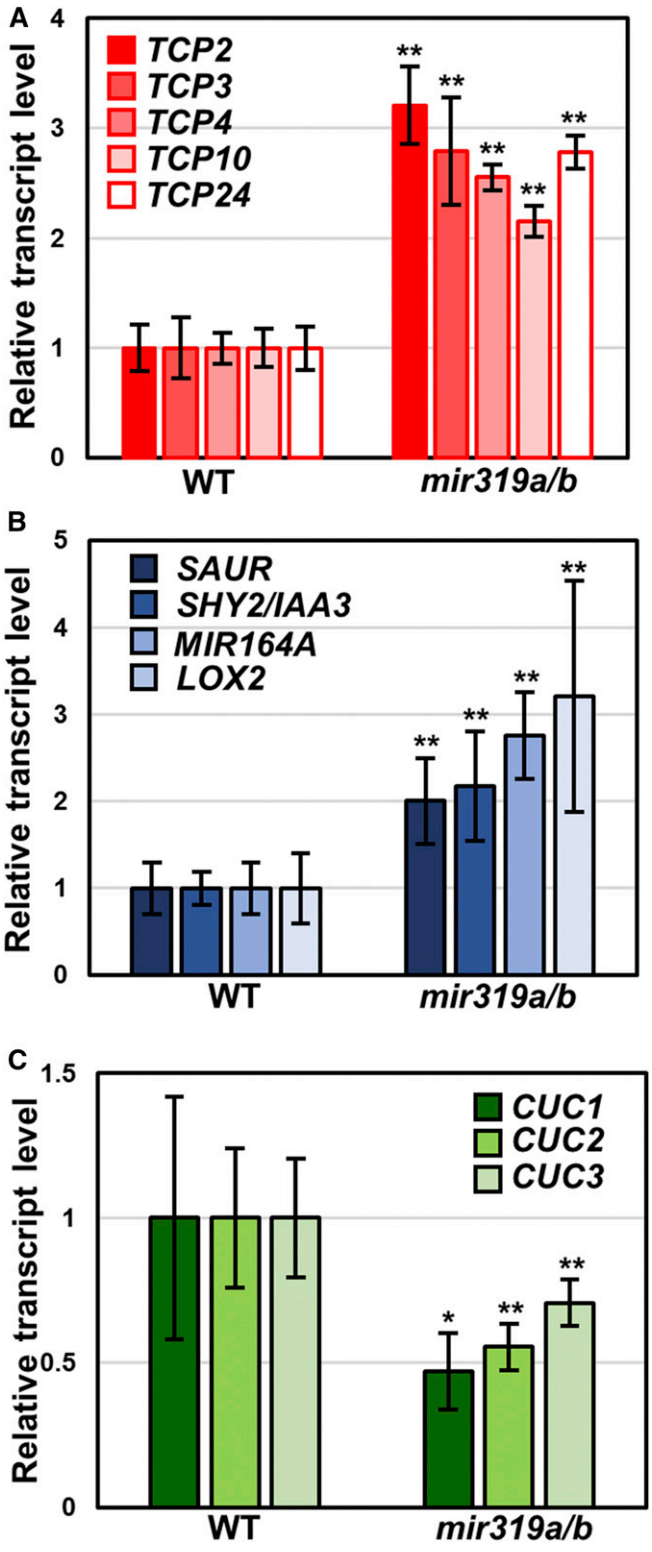

Figure 2. Gene expression analysis of wild-type and *mir319a/b* seedlings. The transcript levels of *TCP* (A), the TCP target (B), and *CUC* (C) genes were analyzed by RT-PCR. The values obtained for wild-type samples were each set at 1. Error bars and asterisks indicate the SD and significant difference of six biological replicates (Student's *t* test; $*P < 0.05$, $**P < 0.01$), respectively.

upregulated in *mir319a/b* seedlings (Fig. 2B). Since TCP transcription factors negatively regulate the expression of the *CUC1*, *CUC2*, and *CUC3* genes, we examined the transcript levels of the *CUC* genes and found their decreased expression in *mir319a/b* seedlings (Fig. 2C). These results demonstrated that the activation of *TCP* genes caused by the *mir319a/b* mutations was able to repress *CUC* gene expression.

The overexpression of *mTCP* genes often results in the fusion of cotyledons and the dysfunctionality of shoot meristems in the transgenic seedlings (Palatnik et al., 2003; Koyama et al., 2007). However, *mir319a*, *mir319b*, and *mir319a/b* showed normal cotyledon morphology (Fig. 3; Supplemental Table S1). Since *mir319a/b* seedlings exhibited a moderate reduction in *CUC* transcript levels, we expected that the *mir319a/b* mutations could induce cotyledon fusion phenotypes in the absence of either of the *CUC* genes. The single *cuc1*, *cuc2*, and *cuc3* mutants are almost normal in their seedling stage due to the functional redundancy of *CUC* genes (Fig. 3; Supplemental Table S1; Aida et al., 1997; Vroemen et al., 2003; Hibara et al., 2006). As expected, all combinations of the double *cuc* and *mir319* mutations induced the cotyledon fusion phenotype to various extents (Fig. 3; Supplemental Table S1). In the severe case, the *cuc* and *mir319* mutations induced a cup-shaped cotyledon-like phenotype, resulting in the inability of rosette leaf formation owing to dysfunction of the shoot meristem, which is observed in the double mutants of *CUC* genes. These results demonstrated that the *mir319a* and *mir319b* mutations enhanced the effect of each single *cuc* mutation on cotyledon development.

CUC2 and *CUC3* are also involved in the formation of serrations at the leaf margin, and mutations in these genes lead to a dramatic reduction in serration of the sixth leaves (Nikovics et al., 2006; Kawamura et al., 2010; Hasson et al., 2011; Bilsborough et al., 2011). Enhanced leaf serration occurs in accordance with the plant's age and is recognized as a developmental consequence of heteroblasty (Poethig, 1997). We expected that leaves developed at the later growth stage would form obvious serrations in *cuc* mutants and could be used to investigate the combined effect of *cuc* and *mir319* mutations on serration formation. As expected, we found a substantial increase in the serration size of the 10th and 14th leaves in comparison to that of the sixth leaves in the wild type (Figs. 1B and 4B). *cuc2* and *cuc3* formed small but obvious serrations in the leaves at the 14th and 10th positions, respectively (Fig. 4, A and B). Under these experimental conditions, *mir319a* and *mir319b* mutations severely decreased the serration size in the *cuc2* and *cuc3* backgrounds. These results demonstrated that *mir319* mutations enhanced the defective effects of *cuc2* and *cuc3* mutations on leaf serration formation.

Gain-of-Function Mutations in *TCP4* Impaired the Cotyledon Boundary and Leaf Serrations

Regarding the gain-of-function analysis of the *TCP* genes, previous studies have used transgenic plants in

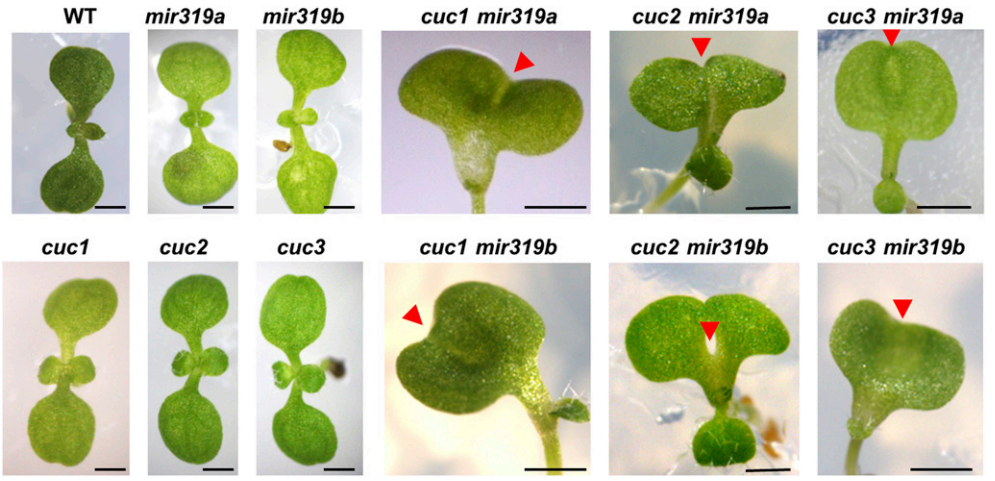

Figure 3. The genetic interactions of *MIR319* and *CUC* genes during cotyledon morphogenesis. The genotypes of wild type, *mir319a*, *mir319b*, *cuc1*, *cuc2*, *cuc3*, and double mutants of various combinations are indicated above the photographs of cotyledons. Triangles show the fusion of cotyledons. Bars = 1 mm

which overexpression constructs of *mTCP* genes are introduced (Palatnik et al., 2003; Koyama et al., 2007; Efroni et al., 2008; Sarvepalli and Nath, 2011). Since TCP transcription factors form homo- and heterodimers (Kosugi and Ohashi 2002, Giraud, et al., 2010; Danisman et al., 2013; Kubota et al., 2017), these overexpression constructs might simultaneously affect several TCP activities. To obtain novel insights in gain-of-function effects of the *TCP* gene and genetic interactions between *TCP* and *CUC* genes in the absence of the overexpression constructs, we used suppressor of *jaw-d* (*soj*) mutants, which possess single-base nucleotide replacements in *TCP4* to be resistant against miR319 (Supplemental Fig. S2; Palatnik et al., 2007). In this study, *soj* mutants were used as gain-of-function mutants for *TCP4* after outcrossing to wild type for the elimination of the *jaw* phenotype due to the enhancer trap (hereafter, the resultant mutants are named *tcp4-d*^{soj6} and *tcp4-d*^{soj8} for discrimination with the outcrossed mutants). As reported previously (Palatnik et al., 2007), we confirmed the mutation in *tcp4-d*^{soj8} at the site corresponding to the reverse position 11 of miR319, where the wild-type *TCP4* mRNAs are cleaved, and *tcp4-d*^{soj6}at the next position of the one in *soj8* (Supplemental Fig. S2). At the seedling stage, *tcp4-d*^{soj6} showed normal morphology whereas *tcp4-d*^{soj8} occasionally induced the cotyledon fusion phenotype (Fig. 5; Supplemental Table S1). Consistent with the role of miR319 in the formation of leaf serrations, *tcp4-d*^{soj6} and *tcp4-d*^{soj8} mutants impaired the formation of leaf serrations (Supplemental Fig. S6A). In inflorescences, the *tcp4-d* mutants exhibited markedly low fertility, as observed in *mir319a/b* and *Pro-35S:mTCP3* plants (Supplemental Figure S6B; Fig. 1E).

To determine the effect of adding the gain-of-function *TCP4* gene to the loss-of-function of *MIR319* genes, *tcp4-d*^{soj6} and *tcp4-d*^{soj8} were individually crossed with *mir319a* and *mir319b*. *tcp4-d*^{soj6} *mir319b* and *tcp4-d*^{soj8} *mir319b* frequently produced the cotyledon fusion phenotype, and the extent of their irregularity was high in comparison to the individual single mutants (Fig. 5; Supplemental Table S1). These results indicated that a synergistic effect existed between the *tcp4-d* and *mir319* mutations. As reported for floral morphogenesis (Nag et al., 2009), *tcp4-d*^{soj6} *mir319a* seedlings exhibited a wild-type-like morphology (Fig. 5; Supplemental Table S1). Since *mir319a* has sequence complementary to *tcp4-d*^{soj6} mRNA (Supplemental Fig. S2), the mutated miR319a may target the *tcp4-d*^{soj6} mRNA in plant cells, leading to a wild-type-like phenotype. The homozygous *tcp4-d*^{soj8} *mir319a* mutant produced no seeds and was a more severely defected phenotype of *tcp4-d*^{soj8}. Since *tcp4-d*^{soj8} *mir319a* mutations led to the replacement of two nucleotides in the complementary sites of miR319 and *TCP4* mRNA (Supplemental Fig. S2), the double mutations might severely impair fertility and embryogenesis in comparison with *tcp4-d*^{soj8}.

As *mir319* mutations enhanced the defective effect of *cuc* genes on cotyledon boundary formation (Fig. 3), we further examined whether *tcp4-d* mutations stimulated the cotyledon fusion phenotype in *cuc* backgrounds. After crossing the *tcp4-d*^{soj6} and *tcp4-d*^{soj8} mutants with the *cuc2* and *cuc3* mutants, all combinations of double *tcp4-d cuc* mutants exhibited the cotyledon fusion phenotype, with remarkably high extent (Fig. 5; Supplemental Table S1). These results demonstrated that the *tcp4-d* mutations enhanced the defective effect of the *cuc2* and *cuc3* mutations on cotyledon boundary formation. Since the *TCP4* and *CUC1* loci are located at close positions on chromosome 1, no homozygous *tcp4-d cuc1* double mutant plants were obtained in this study.

These effects of the gain-of-function *TCP4* gene mutation are consistent with those of the loss-of-function *MIR319* gene mutation. Taken together, our results provide genetic evidence that release of the *TCP* genes from miR319-targeted negative regulation impairs the formation of the cotyledon boundary and leaf serrations.

Mutations in *TCP* Genes Stimulated the Complexity of Leaf Forms

The phylogenetic analysis divided the eight *TCP* genes into two clades, comprising the miR319-targeted

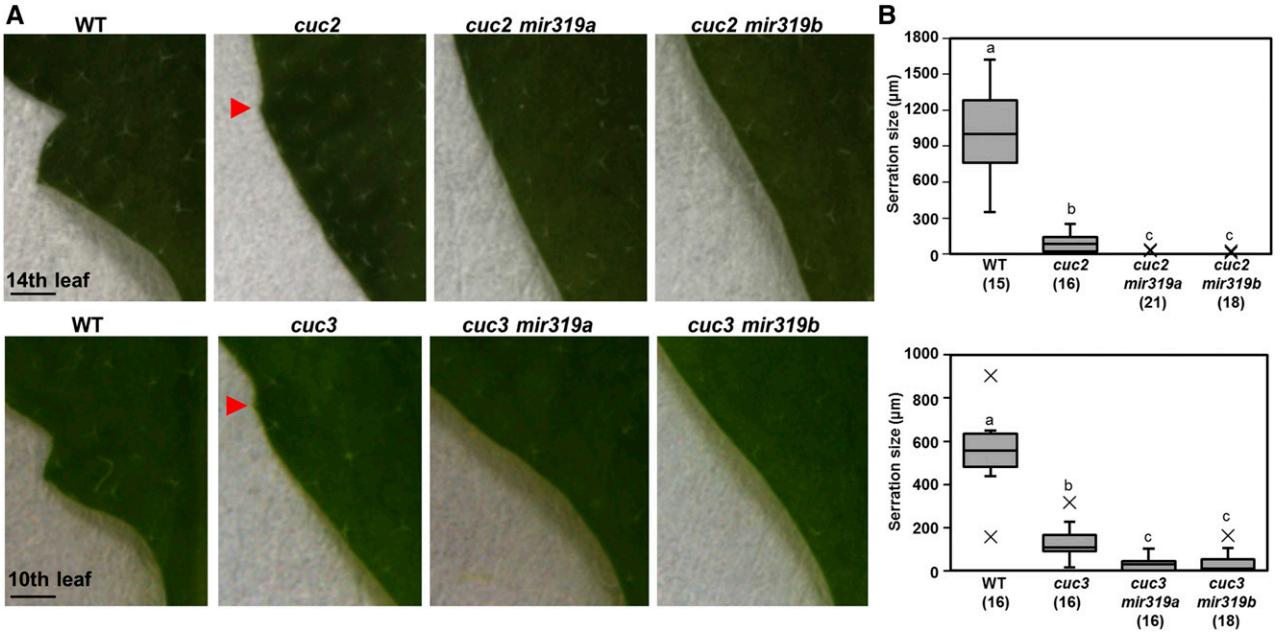

Figure 4. The genetic interactions of *MIR319* and *CUC* genes in the formation of leaf serrations. A, The genotypes of various mutants are indicated above the photographs of leaf serrations. Triangles show the serration at the leaf margin. Bars = 1 mm. B, Quantification of the size of leaf serrations. A detailed description of the box plots is provided in Figure 1B.

and nontargeted *TCP* genes (Supplemental Fig. S1). The involvement of miR319-targeted *TCP* genes in leaf development has been extensively investigated; however, the role of nontarget *TCP* genes, namely, *TCP5*, *TCP13*, and *TCP17*, has not been fully clarified. We previously obtained genetic evidence showing the actions of the *TCP5* and *TCP13* genes with the five miR319-targeted *TCP* genes in the regulation of leaf development (Koyama et al., 2010). To extend our findings, here, we clarified the involvement of *TCP17* in the regulation of leaf development. The T-DNA insertion line of *TCP17* (*tcp17*) was identified and subsequently crossed with *tcp5* and *tcp13* to generate *tcp5 tcp13 tcp17* (*tcp5/13/17*; Supplemental Fig. S7, A and B; Alonso et al., 2003). The *tcp17*, *tcp5/17*, and *tcp5/13/17* mutants generated normal leaves with respect to the wild-type-like margin and surface flatness (Supplemental Fig. S7C), indicating the redundancy of these nontarget *TCP* genes.

In contrast, *tcp3 tcp4 tcp5 tcp10 tcp13 tcp17 (tcp3/4/5/10/13/17)* formed wavy and serrated leaves, and this abnormality was more severe than that of *tcp3 tcp4 tcp10 (tcp3/4/10)* leaves (Fig. 6A; Supplemental Fig. S7D). Therefore, *tcp5/13/17* mutations enhanced the leaf abnormalities caused by the *tcp3 tcp4 tcp10* mutation (Koyama et al., 2010). Consistent with the observation that TCP transcription factors negatively regulate the expression of *CUC* genes, *tcp3/4/10* and *tcp3/4/5/10/13/17* leaves had markedly increased *CUC* transcript levels in comparison with the wild-type leaves (Fig. 6B). In addition, *tcp3/4/10* and *tcp3/4/5/10/13/17* mutants

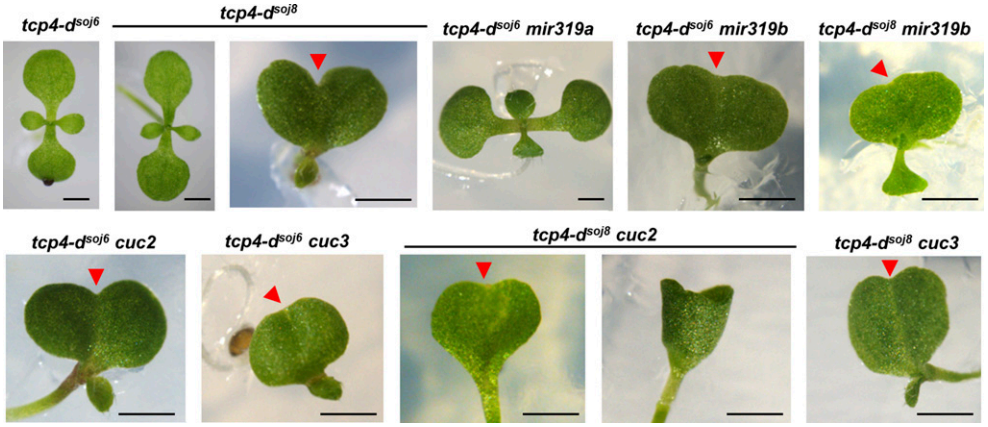

Figure 5. The genetic interactions of *TCP4* and *CUC* genes during cotyledon morphogenesis. The genotypes of mutants are indicated above the photographs of cotyledons. Triangles show the fusion of cotyledons. Bars = 1 mm.

inhibited hypocotyl elongation (Supplemental Fig. S8). This phenotype was in contrast to enhanced hypocotyl elongation due to the ectopic activation of the miR319-targeted *TCP* genes in *mir319a/b* and *Pro-35S:mTCP* plants (Fig. 1, C and D; Palatnik et al., 2003; Koyama et al., 2007).

Furthermore, all eight *TCP* genes were simultaneously downregulated following the introduction of the *Pro-35S:miR319A* gene, which dramatically reduced *TCP2* and *TCP24* transcripts, as reported previously (Palatnik et al., 2003; Koyama et al., 2007; Efroni et al., 2008), in *tcp3/4/5/10/13/17* plants (Supplemental Fig. S7E). The *Pro-35S:miR319A tcp3/4/5/10/13/17* seedlings exhibited ectopic shoot meristem formation and usually produced several leaves with a jagged and wavy shape but later ceased their development (Fig. 6C). The dysfunctionality in shoot meristems of *Pro-35S:miR319A tcp3/4/5/10/13/17* seedlings was consistent with the phenotype of the transgenic plants expressing chimeric *TCP* repressor genes (Koyama et al., 2007). Collectively, the results of our analyses provided genetic evidence that, although *TCP5*, *TCP13*, and *TCP17* genes were not targets of miR319, these genes acted in concert with the miR319-targeted *TCP* genes during leaf development, hypocotyl elongation, and shoot meristem formation.

Roles of *miR319* and *TCP* Genes in the Onset of Leaf Senescence

A previous study found that *Pro-35S:mTCP4* plants stimulate leaf senescence, whereas ectopic accumulation of miR319 delays leaf senescence (Schommer et al., 2008; Koyama 2014). In line with this, *Pro-35S:mTCP3*, *tcp4-d*^{soj8} and *mir319a/b* exhibited precocious yellowing of leaves. *mir319a/b* reduced the chlorophyll content, a marker of leaf senescence, earlier than in wild type (Fig. 7, A and B). Conversely, the *tcp3/4/10* and *tcp3/4/5/10/13/17* mutants exhibited delayed yellowing of leaves and sustained the chlorophyll content (Fig. 7, C and D). The *tcp3/4/5/10/13/17* mutant leaves remained green for the longest compared with the other genotypes

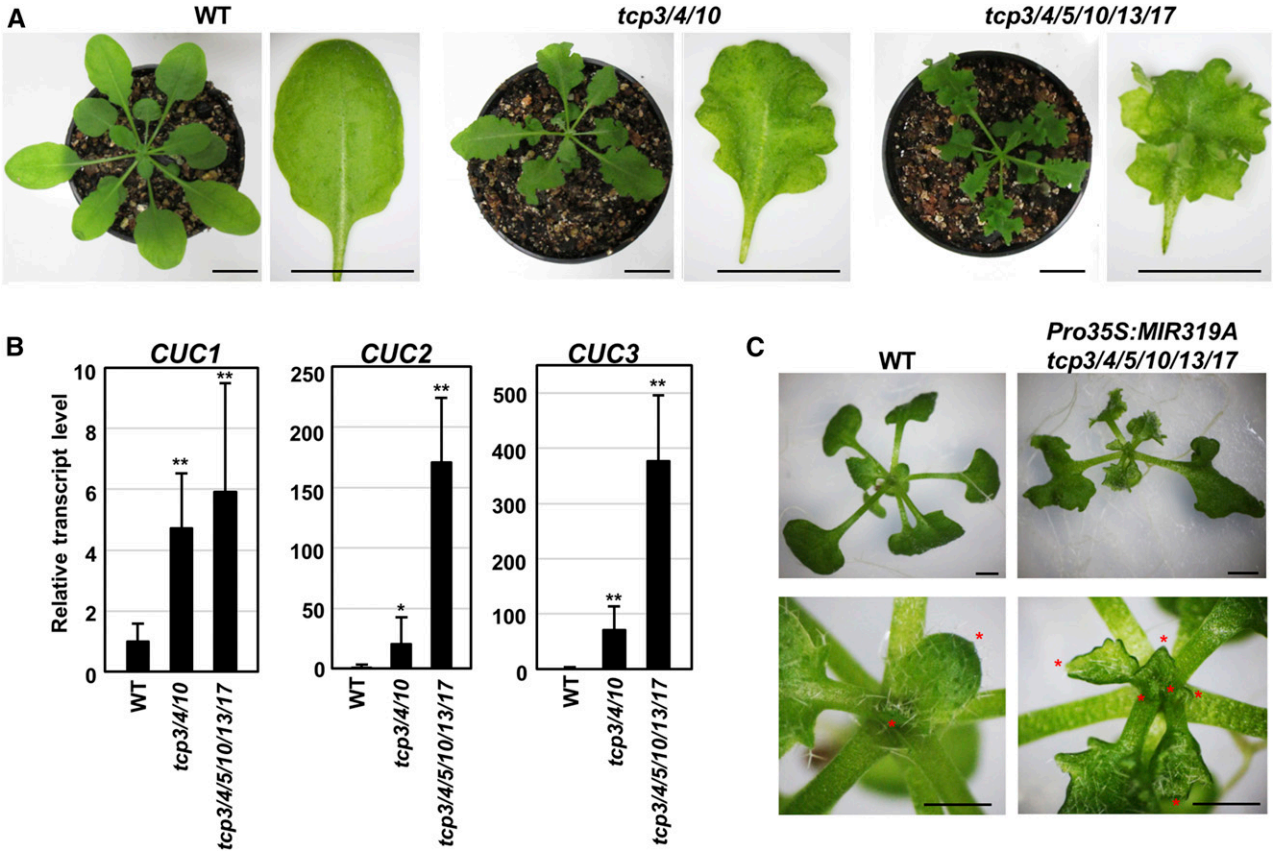

Figure 6. Effects of multiple mutants of the *TCP* gene subfamily on leaf morphogenesis. A, Photographs of rosettes and the sixth leaves of wild-type, *tcp3/4/10*, and *tcp3/4/5/10/13/17* plants. Bars = 1 cm. B, Expression analysis of *CUC1*, *CUC2*, and *CUC3* genes. The levels of *CUC1*, *CUC2*, and *CUC3* transcripts determined by RT-PCR analysis were normalized using levels of the *UBQ1* transcript, and the values for the wild type were set at 1. Error bars and asterisks indicate the SD and significant difference of six biological replicates (Student's *t* test; $*P < 0.05$, $**P < 0.01$), respectively. C, Photographs of the rosettes (top) and shoot apexes (bottom) of 15-d-old wild-type and *Pro-35S:MIR319A tcp3/4/5/10/13/17* plants. Asterisks in the bottom panels indicate initiating leaves. Bars = 1 mm.

investigated. These results demonstrate that the differential *TCP* activities caused by mutations in the *MIR319* and *TCP* genes altered the pace of leaf senescence.

DISCUSSION

The interplay of miRNA networks, including miR319 and *TCP* genes, is a central process in the regulation of leaf development (Chitwood and Sinha, 2016; Rodriguez et al., 2016; Fouracre and Poethig, 2016). To obtain genetic insights into the role of miR319 in the regulation of leaf development, we generated loss-of-function alleles for miR319 and conducted a phenotypic analysis of *mir319a/b*. Since *mir319a* and *mir319b* mutations moderately reduced the size of leaf serrations and the *mir319a/b* mutation almost suppressed their formation, our results indicate that these *MIR319* genes act in a largely quantitative manner during leaf serration formation. Consistently, *mir319a/b* double mutation increased transcripts of the five *TCP* genes, but each single mutation caused mild effects on the transcript levels (Fig. 2). This quantitative mode of miR319 action is different from that of miR164, which targets a family of transcription factor genes including *CUC1* and *CUC2*, since the *MIR164A* gene, among the three *MIR164* genes, specifically contributes to the regulation of leaf serration formation (Nikovics et al., 2006; Sieber et al., 2007). Since loss-of-function alleles for *MIR319C* were not available from, e.g., public resource centers, the contribution of *MIR319C* in leaf development remains to be clarified.

Our results demonstrate that combined mutations in the miR319-mediated regulatory pathway impair the cotyledon boundary. *mir319a/b* and *tcp4-d*^{soj6} mutations exhibited normal cotyledon morphology, and the *tcp4-d*^{soj8} mutant weakly exhibited a cotyledon fusion phenotype. In contrast, the combination of *tcp4-d*^{soj6} *mir319b* and *tcp4-d*^{soj8} *mir319b* mutations induced the cotyledon fusion phenotype. Therefore, these two independent mutations in the miR319-mediated regulatory pathway act synergistically to inhibit cotyledon boundary formation, whereas the contribution of each mutation alone is relatively small. Compared with the

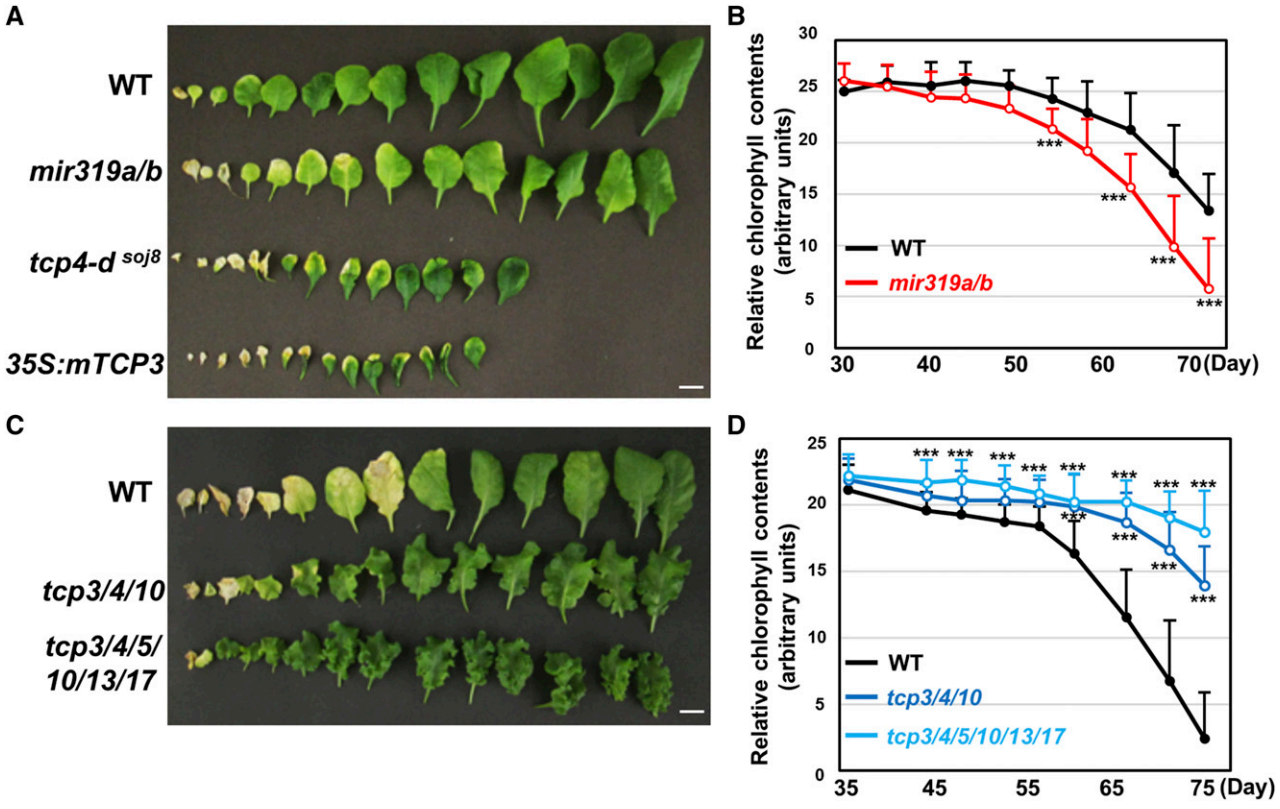

Figure 7. Roles of *MIR319* and *TCP* genes in the onset of leaf senescence. A, A photograph showing wild-type, *mir319a/b*, *tcp4-d*^{soj8}, and *Pro-35S:mTCP3* leaves 65 d after germination. The oldest 14 leaves from the indicated genotypes are presented. Bar = 1 cm. B, The relative chlorophyll content of the sixth leaves of wild-type and *mir319a/b* plants at indicated ages. The error bars and asterisks indicate the SD and significant difference of 16 biological replicates (Student's *t* test; ***$P < 0.001$), respectively. C, A photograph showing wild-type, *tcp3/4/10*, and *tcp3/4/5/10/13/17* leaves 75 d after germination. The detailed description is represented in A. D, The relative chlorophyll content of the sixth leaves of wild-type, *tcp3/4/10*, and *tcp3/4/5/10/13/17* leaves at the indicated ages. A detailed description of SPAD values is represented in B.

leaf serrations, the cotyledon boundary is likely to be tolerant against the genetic mutations in the miR319-mediated regulatory pathway. Consistently, a tomato homozygous *lanceolate* mutation impairs its cotyledons and shoot meristem, while a heterozygous mutation is sufficient for conversion of the originally complex leaf form into a simple form (Mathan and Jenkins, 1962; Settler, 1964; Ori et al., 2007).

An important finding of this study is that mutations in the miR319-mediated regulatory pathway enhance the defective effects of *cuc* mutations on the phenotype. The crossing of *mir319a*, *mir319b*, *tcp4-d*^{soj6}, and *tcp4-d*^{soj8} with one of three *cuc* mutations remarkably reduced the size of leaf serrations and induced the cotyledon-fusion phenotype to a considerably high degree. In particular, the *cuc2* mutation in combination with *tcp4-d*^{soj6} or *tcp4-d*^{soj8} resulted in the highest frequency of the cotyledon-fusion phenotype. Since the contribution of *cuc2* to the cotyledon-fusion phenotype is greater than that of *cuc1* and *cuc3* (Hibara et al., 2006), our results reflect the main contribution of the *cuc2* mutation to cotyledon development. Furthermore, a mutation in one of the *CUC* genes substantially recovers the phenotype of irregularly serrated cotyledons induced by the chimeric *TCP3* repressor gene in transgenic plants (Koyama et al., 2007). The *cuc2* mutation suppresses the morphology of the serrated leaf margin caused by the overexpression of the *MIR319* gene (Hasson et al., 2011). Overall, these observations provide the genetic evidence of the miR319-mediated control of the *TCP* genes on the regulation of *CUC* genes without the overexpression constructs of *TCP* and *MIR319* genes and are consistent with the previous reports of the negative actions of TCP transcription factors on the activities of *CUC* genes (Fig. 8A; Koyama et al., 2007, 2010; Rubio-Somoza et al., 2014).

Regarding the function of *TCP* genes, we demonstrate that, in concert with the miR319-targeted *TCP* genes, nontargeted *TCP* genes regulate leaf development in a highly redundant manner. Reasonably, increased expression of the *CUC* transcripts in triple and sextuple *tcp* mutants is associated with drastically complex leaf forms. Extending previous reports on the isolation of *mir319*- and *tcp*-related single mutants (Koyama et al., 2007, 2010; Palatnik et al., 2007; Nag et al., 2009), we have generated a series of mutants possessing *TCP* genes with varying levels of activation. Inhibition of *TCP* genes increases the complexity of leaves, but conversely, their activation simplifies the complexity of leaves (Fig. 8B). In addition to *tcp5/13/17*, all single and double mutants tested exhibited the wild-type-like form (this work; Koyama et al., 2007, 2010). Therefore, in addition to control by miR319, the extreme redundancy of *TCP* genes underlies the generation of differentially modified leaf forms. Our results showing the dysfunctionalities of shoot meristems in *Pro-35S:miR319A tcp3/4/5/10/13/17* shed light on another important aspect of this redundancy. We can speculate that, if a certain *TCP* gene centralizes roles played by all eight *TCP* genes, a genetic mutation in this single gene would be lethal.

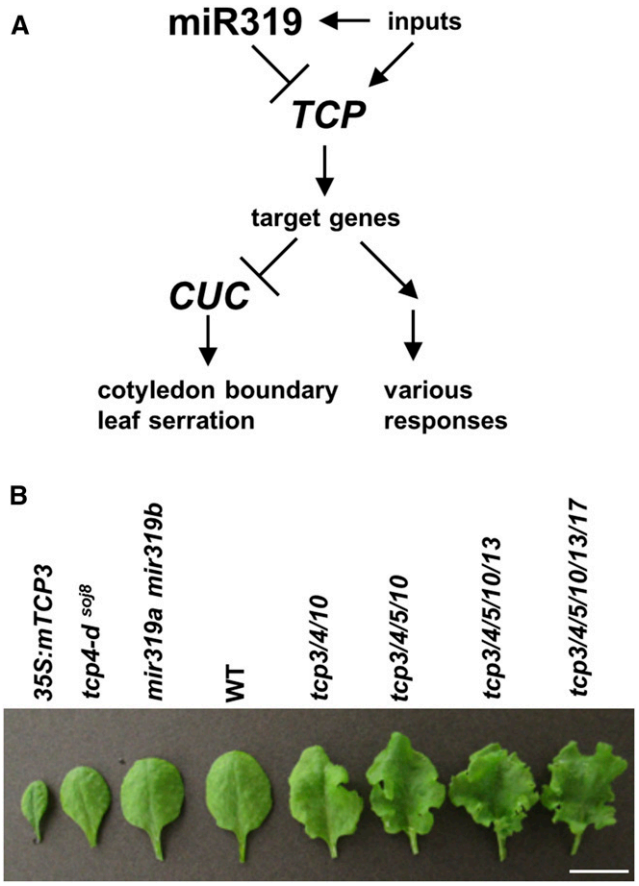

Figure 8. Roles of miR319 and *TCP* genes in leaf morphogenesis. A, A schematic representation of the action of *MIR319* and *TCP* genes. T bars and arrows indicate negative and positive relationships, respectively. miR319 posttranscriptionally represses its target *TCP* genes downstream of developmental inputs. The miR319-taregeted and nontargeted TCP transcription factors cooperatively regulate their downstream genes, such as *CUC* genes, for the cotyledon boundary and leaf serration formation and for other physiological responses. B, Differentially modified leaf forms induced by *TCP* genes activated to various degrees. The sixth leaves of the indicated genotypes are arranged in a line. Bars = 1 cm. Furthermore, details on the mutants are as follows: *mir319a/b, tcp3/4/5/10/13/17* (this work), *Pro-35S:mTCP3* (Koyama et al., 2007), *tcp4-d* (Palatnik et al., 2007; this work), and *tcp3/4/10, tcp3/4/5/10, tcp3/4/5/10/13* (Koyama et al., 2010).

In the above context, miR319 finely tunes the expression of its target *TCP* genes downstream of unidentified developmental inputs. The overlapping but slightly different expression pattern of the miR319-targeted and nontargeted *TCP* genes in cotyledons and leaves confers robustness to the regulation of leaf development (Fig. 8A; Palatnik et al., 2003, Koyama et al., 2007; Alvarez et al., 2016). With respect to the downstream network that includes *CUC* genes, TCP transcription factors directly regulate genes related to the functions of miRNA, differentiation, cell cycling, and plant hormones (Fig. 8A; Schommer et al., 2008, 2014; Koyama et al., 2010; Rodriguez et al., 2010; Efroni et al., 2013; Danisman et al., 2013; Rubio-Somoza and Weigel, 2013; Ballester et al., 2015; Challa et al., 2016).

Since TCP transcription factors can form homo- and heterodimers within and between the subfamilies (Kosugi and Ohashi, 2002; Giraud, et al., 2010; Danisman et al., 2013; Kubota et al., 2017), it is possible that each TCP shares its downstream genes in an overlapping manner. In addition, TCP-interacting proteins seem to participate in the modification of protein levels (Li et al., 2012; Efroni et al., 2013; Tao et al., 2013; Li and Zachgo 2013; Ho and Weigel 2014; Wei et al., 2015). However, a view of the whole network intertwined by *TCP* genes remains to be clarified.

Furthermore, miR319 and *TCP* genes gradually regulate leaf senescence. *mir319a/b* induced *LOX2*, whereas *tcp3/4/10* and *tcp3/4/5/10/13/17* inhibited *LOX2*. Therefore, *LOX2*, which encodes a jasmonate biosynthesis enzyme and accelerates leaf senescence may mediate these senescence-related phenotypes (He et al., 2002; Schommer et al., 2008). In addition, large-scale expression analyses illustrate the down-regulation of *TCP* genes in association with leaf senescence but do not show any difference in the miR319 level during leaf senescence (Breeze et al., 2011; Thatcher et al., 2015). Thus, miR319 appears to finely tune the activity of *TCP* genes in order to set the appropriate pace of leaf senescence rather than exclusively inhibit their activity in the senescent leaves.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*; Columbia-0) was used throughout the study unless otherwise indicated. *mir319a*^{123} (*Landsberg erecta*; Nag et al., 2009) was crossed three times with Columbia-0 before use for standardization of the genetic background. *mir319b* was obtained from the GABI_KAT collection (Rosso et al., 2003). *soj6* and *soj8* (Palatnik et al., 2007) were outcrossed twice with Col-0 to eliminate an enhancer trap construct. *cuc1-5*, *cuc2-3*, and *cuc3-105* have been described previously (Hibara et al., 2006). To generate the double mutants indicated in the text, the respective single mutants, in appropriate combinations, were crossed. *tcp17* was obtained from the Arabidopsis Bioresource Center (Alonso et al., 2003) and crossed with *tcp5* and *tcp13* (Koyama et al., 2010) for the generation of *tcp5 tcp17* and *tcp13 tcp17*, respectively. These double *tcp* mutants were crossed for the generation of the *tcp5/13/17* mutant. To generate the sextuple *tcp* mutant, we first established triple mutants of *tcp3/5/17*, *tcp3/4/5*, and *tcp3/5/13*, using the alleles established (this work; Koyama et al., 2007, 2010). Next, independent crossings of *tcp3/4/5* with *tcp3/5/13* and *tcp3/5/17* resulted in the generation of *tcp3/4/5/13* and *tcp3/4/5/17*, respectively. *tcp3/4/5/13* and *tcp3/4/5/17* were further crossed for the generation of *tcp3/4/5/13/17*. Finally, *tcp3/4/5/13/17* and *tcp3/4/5/10/13* (Koyama et al., 2010) were crossed for the generation of *tcp3/4/5/10/13/17*. To determine plant genotypes, the single-base nucleotide changes corresponding to *mir319a*^{123}, *soj6*, and *soj8* were determined by sequencing of the respective genomic regions. Mutations due to the T-DNA insertions were identified by genomic PCR using the appropriate sets of T-DNA- and gene-specific primers, and RT-PCR using gene-specific primers (Supplemental Table S2).

For the growth of plants, seeds were sterilized by sodium hypochlorite and grown on a plate containing half-strength Murashige and Skoog salts, 0.5 g/L MES, and 5 g/L Suc under 16-h-light/8-h-dark conditions, unless otherwise indicated. The *Pro-35S:MIR319A* construct was described previously (Koyama et al., 2007) and was introduced into *tcp3/4/5/10/13/17* for the generation of *Pro-35S:miR319A tcp3/4/5/10/13/17* plants using *Agrobacterium tumefaciens*-mediated gene transformation (Koyama et al., 2010).

Gene Expression

Plant tissues were harvested 9 to 11 h after removal from the dark to minimize potential effects of circadian rhythms on the transcriptional profile of *TCP* and the target genes (Giraud et al., 2010). Aliquots of total RNA were prepared from plant tissues, reverse-transcribed using an oligo(dT) primer, and subjected to real-time PCR analysis with a CFX96 real-time PCR system (Bio-Rad) using appropriate sets of primers (Supplemental Table S2). A standard curve derived from the reference sample was used to confirm the correct amplification efficiency of the primer pairs and to calculate the transcript levels of the genes of interest. The relative values of the transcript levels were normalized to that of *UBQ1*. Similar patterns of relative values were obtained when the results were normalized using another internal control gene, *PP2AA3*.

Light Microscopy Analysis

For the observation of cotyledons, seedlings were grown on a plate for 7 d. For observation of leaf serrations, each experiment was conducted using either set of genotypes as indicated in Figures 1A and 4A. Seeds were sowed on soil and seedlings germinated were individually transferred to specialized plastic pots (Arasystem 360; Betatech). The sets of genotypes were arranged side by side in a platter pooled with water. Plants were nourished with 1 mL of liquid fertilizer (HYPONeX) once a week and grown under 12-h-light/12-h-dark conditions, in which plants prolonged the vegetative growth phase. The 10th and 14th leaves were detached at 5 and 6 weeks old, respectively. Detached leaves were covered with a conventional slide grass, and their serration in the margin of the basal end was photographed using a M205 stereomicroscope (Leica Microsystems). The scale of a serration from its tip to base end was measured from using LAS AF software (Leica Microsystems). Statistical analysis and box-plot development were performed using BellCurve_for_Excel software with the default settings (https://bellcurve.jp/ex/). One-way ANOVA was performed, followed by the Tukey-Kramer method for multiple comparisons. $P < 0.05$ was considered statistically significant.

Senescence Assay

Plants were grown on soil under a 12-h-light/12-h-dark cycle. To calculate the relative chlorophyll contents, the sixth leaves of the individual genotypes were measured using a SPAD 502-devise (Konica-Minolta; Ling et al., 2011), and the SPAD values were averaged from 16 biological replicates of arbitrary units.

Accession Numbers

Sequence data from this article have been deposited in the GenBank/EMBL data libraries under the following accession numbers: TCP2 (AT4G18390), TCP3 (AT1G53230), TCP4 (AT3G15030), TCP5 (AT5G60970), TCP10 (AT2G31070), TCP13 (AT3G02150), TCP17 (AT5G08070), MIR319A (AT4G23713), MIR319B (AT5G41663), MIR319C (AT2G40805), MIR164A (At2g47585), INDOLE-3-ACETIC ACID3/SHORT HYPOCOTYL2 (At1g04240), CUC1 (At3g15170), CUC2 (At5g53950), and CUC3 (At1g76420).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Phylogeny of *CINCINNATA*-like *TCP* genes.

Supplemental Figure S2. Alignment of the wild-type and mutated *MIR319A*, *TCP3*, and *TCP4* genes with miR319.

Supplemental Figure S3. Expression of the *MIR319* genes in various organs.

Supplemental Figure S4. Identification of *mir319b*.

Supplemental Figure S5. Expression of the miR319-targeted *TCP* genes in *mir319a* and *mir319b* seedlings.

Supplemental Figure S6. The morphology of leaves and inflorescences in *tcp-4d* mutants.

Supplemental Figure S7. Generation of *tcp17* and related mutants.

Supplemental Figure S8. Inhibition of hypocotyl elongation in *tcp3/4/10* and *tcp3/4/5/10/13/17* plant growth under light conditions.

Supplemental Table S1. The appearance of the cotyledon fusion phenotype in various genotypes.

Supplemental Table S2. List of primers used in this study.

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LITERATURE CITED

Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M (1997) Genes involved in organ separation in Arabidopsis: An analysis of the cup-shaped cotyledon mutant. Plant Cell **9:** 841–857

Aida M, Tasaka M (2006) Genetic control of shoot organ boundaries. Curr Opin Plant Biol **9:** 72–77

Alvarez JP, Furumizu C, Efroni I, Eshed Y, Bowman JL (2016) Active suppression of a leaf meristem orchestrates determinate leaf growth. eLife **5:** 1–17

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301:** 653–657

Ballester P, Navarrete-Gómez M, Carbonero P, Oñate-Sánchez L, Ferrándiz C (2015) Leaf expansion in Arabidopsis is controlled by a TCP-NGA regulatory module likely conserved in distantly related species. Physiol Plant **155:** 21–32

Bar M, Ori N (2014) Leaf development and morphogenesis. Development **141:** 4219–4230

Bilsborough GD, Runions A, Barkoulas M, Jenkins HW, Hasson A, Galinha C, Laufs P, Hay A, Prusinkiewicz P, Tsiantis M (2011) Model for the regulation of *Arabidopsis thaliana* leaf margin development. Proc Natl Acad Sci USA **108:** 3424–3429

Breeze E, Harrison E, McHattie S, Hughes L, Hickman R, Hill C, Kiddle S, Kim YS, Penfold CA, Jenkins D, et al (2011) High-resolution temporal profiling of transcripts during Arabidopsis leaf senescence reveals a distinct chronology of processes and regulation. Plant Cell **23:** 873–894

Challa KR, Aggarwal P, Nath U (2016) Activation of YUCCA5 by the transcription factor TCP4 integrates developmental and environmental signals to promote hypocotyl elongation in Arabidopsis. Plant Cell **28:** 2117–2130

Chitwood DH, Sinha NR (2016) Evolutionary and environmental forces sculpting leaf development. Curr Biol **26:** R297–R306

Danisman S, van Dijk ADJ, Bimbo A, van der Wal F, Hennig L, de Folter S, Angenent GC, Immink RGH (2013) Analysis of functional redundancies within the Arabidopsis TCP transcription factor family. J Exp Bot **64:** 5673–5685

Efroni I, Blum E, Goldshmidt A, Eshed Y (2008) A protracted and dynamic maturation schedule underlies Arabidopsis leaf development. Plant Cell **20:** 2293–2306

Efroni I, Han S-K, Kim HJ, Wu M-F, Steiner E, Birnbaum KD, Hong JC, Eshed Y, Wagner D (2013) Regulation of leaf maturation by chromatin-mediated modulation of cytokinin responses. Dev Cell **24:** 438–445

Fouracre JP, Poethig RS (2016) The role of small RNAs in vegetative shoot development. Curr Opin Plant Biol **29:** 64–72

Gion K, Suzuri R, Shikata M, Mitsuda N, Oshima Y, Koyama T, Ohme-Takagi M, Ohtsubo N, Tanaka Y (2011) Morphological changes of *Rosa x hybrida* by a chimeric repressor of Arabidopsis TCP3. Plant Biotechnol **28:** 149–152

Giraud E, Ng S, Carrie C, Duncan O, Low J, Lee CP, Van Aken O, Millar AH, Murcha M, Whelan J (2010) TCP transcription factors link the regulation of genes encoding mitochondrial proteins with the circadian clock in *Arabidopsis thaliana*. Plant Cell **22:** 3921–3934

Hasson A, Plessis A, Blein T, Adroher B, Grigg S, Tsiantis M, Boudaoud A, Damerval C, Laufs P (2011) Evolution and diverse roles of the CUP-SHAPED COTYLEDON genes in Arabidopsis leaf development. Plant Cell **23:** 54–68

He Y, Fukushige H, Hildebrand DF, Gan S (2002) Evidence supporting a role of jasmonic acid in Arabidopsis leaf senescence. Plant Physiol **128:** 876–884

Hibara K, Karim MR, Takada S, Taoka K, Furutani M, Aida M, Tasaka M (2006) Arabidopsis CUP-SHAPED COTYLEDON3 regulates postembryonic shoot meristem and organ boundary formation. Plant Cell **18:** 2946–2957

Hiratsu K, Matsui K, Koyama T, Ohme-Takagi M (2003) Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in Arabidopsis. Plant J **34:** 733–739

Ho WWH, Weigel D (2014) Structural features determining flower-promoting activity of Arabidopsis FLOWERING LOCUS T. Plant Cell **26:** 552–564

Ichihashi Y, Tsukaya H (2015) Behavior of leaf meristems and their modification. Front Plant Sci **6:** 1060

Kawamura E, Horiguchi G, Tsukaya H (2010) Mechanisms of leaf tooth formation in Arabidopsis. Plant J **62:** 429–441

Kosugi S, Ohashi Y (2002) DNA binding and dimerization specificity and potential targets for the TCP protein family. Plant J **30:** 337–348

Koyama T (2014) The roles of ethylene and transcription factors in the regulation of onset of leaf senescence. Front Plant Sci **5:** 650

Koyama T, Furutani M, Tasaka M, Ohme-Takagi M (2007) TCP transcription factors control the morphology of shoot lateral organs via negative regulation of the expression of boundary-specific genes in Arabidopsis. Plant Cell **19:** 473–484

Koyama T, Mitsuda N, Seki M, Shinozaki K, Ohme-Takagi M (2010) TCP transcription factors regulate the activities of ASYMMETRIC LEAVES1 and miR164, as well as the auxin response, during differentiation of leaves in Arabidopsis. Plant Cell **22:** 3574–3588

Koyama T, Ohme-Takagi M, Sato F (2011) Generation of serrated and wavy petals by inhibition of the activity of TCP transcription factors in *Arabidopsis thaliana*. Plant Signal Behav **6:** 697–699

Kubota A, Ito S, Shim JS, Johnson RS, Song YH, Breton G, Goralogia GS, Kwon MS, Laboy Cintrón D, Koyama T, et al (2017) TCP4-dependent induction of CONSTANS transcription requires GIGANTEA in photoperiodic flowering in Arabidopsis. PLoS Genet **13:** e1006856

Li Z, Li B, Shen WH, Huang H, Dong A (2012) TCP transcription factors interact with AS2 in the repression of class-I KNOX genes in *Arabidopsis thaliana*. Plant J **71:** 99–107

Li S, Zachgo S (2013) TCP3 interacts with R2R3-MYB proteins, promotes flavonoid biosynthesis and negatively regulates the auxin response in *Arabidopsis thaliana*. Plant J **76:** 901–913

Ling Q, Huang W, Jarvis P (2011) Use of a SPAD-502 meter to measure leaf chlorophyll concentration in *Arabidopsis thaliana*. Photosynth Res **107:** 209–214

Lopez JA, Sun Y, Blair PB, Mukhtar MS (2015) TCP three-way handshake: Linking developmental processes with plant immunity. Trends Plant Sci **20:** 238–245

Maugarny A, Gonçalves B, Arnaud N, Laufs P (2016) CUC transcription factors: To the meristem and beyond. *In* DH Gonzalez, ed, Plant Transcription Factors. Academic Press, London, pp 229–247

Mathan D, Jenkins J (1962) A morphogenic study of lanceolate, a leaf-shape mutant in the tomato. Am J Bot **49:** 504–514

Nag A, King S, Jack T (2009) miR319a targeting of TCP4 is critical for petal growth and development in Arabidopsis. Proc Natl Acad Sci USA **106:** 22534–22539

Narumi T, Aida R, Koyama T, Yamaguchi H, Sasaki K, Shikata M, Nakayama M, Ohme-Takagi M, Ohtsubo N (2011) Arabidopsis chimeric TCP3 repressor produces novel floral traits in *Torenia fournieri* and *Chrysanthemum morifolium*. Plant Biotechnol **28:** 131–140

Nath U, Crawford BCW, Carpenter R, Coen E (2003) Genetic control of surface curvature. Science **299:** 1404–1407

Nicolas M, Cubas P (2016) TCP factors: New kids on the signaling block. Curr Opin Plant Biol **33:** 33–41

Nikovics K, Blein T, Peaucelle A, Ishida T, Morin H, Aida M, Laufs P (2006) The balance between the MIR164A and CUC2 genes controls leaf margin serration in Arabidopsis. Plant Cell **18:** 2929–2945

Ono M, Hiyama S, Higuchi Y, Kamada H, Nitasaka E, Koyama T, Mitsuda N, Ohme-Takagi M, Sage-Ono K (2012) Morphological changes in *Ipomoea nil* using chimeric repressors of Arabidopsis TCP3 and TCP5. Plant Biotechnol **29:** 457–463

Ori N, Cohen AR, Etzioni A, Brand A, Yanai O, Shleizer S, Menda N, Amsellem Z, Efroni I, Pekker I, et al (2007) Regulation of LANCEOLATE by miR319 is required for compound-leaf development in tomato. Nat Genet **39:** 787–791

Palatnik JF, Allen E, Wu X, Schommer C, Schwab R, Carrington JC, Weigel D (2003) Control of leaf morphogenesis by microRNAs. Nature **425:** 257–263

Palatnik JF, Wollmann H, Schommer C, Schwab R, Boisbouvier J, Rodriguez R, Warthmann N, Allen E, Dezulian T, Huson D, et al (2007) Sequence and

expression differences underlie functional specialization of Arabidopsis microRNAs miR159 and miR319. Dev Cell **13:** 115–125

Poethig RS (1997) Leaf morphogenesis in flowering plants. Plant Cell **9:** 1077–1087

Rodriguez RE, Mecchia MA, Debernardi JM, Schommer C, Weigel D, Palatnik JF (2010) Control of cell proliferation in *Arabidopsis thaliana* by microRNA miR396. Development **137:** 103–112

Rodriguez RE, Schommer C, Palatnik JF (2016) Control of cell proliferation by microRNAs in plants. Curr Opin Plant Biol **34:** 68–76

Rosso MG, Li Y, Strizhov N, Reiss B, Dekker K, Weisshaar B (2003) An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. Plant Mol Biol **53:** 247–259

Rubio-Somoza I, Weigel D (2013) Coordination of flower maturation by a regulatory circuit of three microRNAs. PLoS Genet **9:** e1003374

Rubio-Somoza I, Zhou CM, Confraria A, Martinho C, von Born P, Baena-Gonzalez E, Wang JW, Weigel D (2014) Temporal control of leaf complexity by miRNA-regulated licensing of protein complexes. Curr Biol **24:** 2714–2719

Sarvepalli K, Nath U (2011) Hyper-activation of the TCP4 transcription factor in *Arabidopsis thaliana* accelerates multiple aspects of plant maturation. Plant J **67:** 595–607

Sasaki K, Yamaguchi H, Kasajima I, Narumi T, Ohtsubo N (2016) Generation of novel floral traits using a combination of floral organ-specific promoters and a chimeric repressor in *Torenia fournieri* Lind. Plant Cell Physiol **57:** 1319–1331

Schommer C, Debernardi JM, Bresso EG, Rodriguez RE, Palatnik JF (2014) Repression of cell proliferation by miR319-regulated TCP4. Mol Plant **7:** 1533–1544

Schommer C, Palatnik JF, Aggarwal P, Chételat A, Cubas P, Farmer EE, Nath U, Weigel D (2008) Control of jasmonate biosynthesis and senescence by miR319 targets. PLoS Biol **6:** e230

Settler R (1964) Dosage effects of the Lanceolate gene in tomato. Am J Bot **51:** 253–264

Shleizer-Burko S, Burko Y, Ben-Herzel O, Ori N (2011) Dynamic growth program regulated by LANCEOLATE enables flexible leaf patterning. Development **138:** 695–704

Sieber P, Wellmer F, Gheyselinck J, Riechmann JL, Meyerowitz EM (2007) Redundancy and specialization among plant microRNAs: role of the MIR164 family in developmental robustness. Development **134:** 1051–1060

Tanaka Y, Yamamura T, Oshima Y, Mitsuda N, Koyama T, Ohme-Takagi M, Terakawa T (2011) Creating ruffled flower petals in *Cyclamen persicum* by expression of the chimeric cyclamen TCP repressor. Plant Biotechnol **28:** 141–147

Tao Q, Guo D, Wei B, Zhang F, Pang C, Jiang H, Zhang J, Wei T, Gu H, Qu LJ, et al (2013) The TIE1 transcriptional repressor links TCP transcription factors with TOPLESS/TOPLESS-RELATED corepressors and modulates leaf development in Arabidopsis. Plant Cell **25:** 421–437

Thatcher SR, Burd S, Wright C, Lers A, Green PJ (2015) Differential expression of miRNAs and their target genes in senescing leaves and siliques: Insights from deep sequencing of small RNAs and cleaved target RNAs. Plant Cell Environ **38:** 188–200

Todesco M, Rubio-Somoza I, Paz-Ares J, Weigel D (2010) A collection of target mimics for comprehensive analysis of microRNA function in *Arabidopsis thaliana*. PLoS Genet **6:** e1001031

Vroemen CW, Mordhorst AP, Albrecht C, Kwaaitaal MACJ, de Vries SC (2003) The CUP-SHAPED COTYLEDON3 gene is required for boundary and shoot meristem formation in Arabidopsis. Plant Cell **15:** 1563–1577

Wei B, Zhang J, Pang C, Yu H, Guo D, Jiang H, Ding M, Chen Z, Tao Q, Gu H, et al (2015) The molecular mechanism of sporocyteless/nozzle in controlling Arabidopsis ovule development. Cell Res **25:** 121–134