

Role of MicroRNA miR319 in Plant Development

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Abstract Originally identified in a genetic screen, microRNA miR319 regulates transcription factors of the *TCP* family. The balance between miR319 and its targets controls leaf morphogenesis and several other plant developmental processes. High levels of miR319 or low *TCP* activity causes an excess of cell proliferation that generates a crinkled simple leaf in *Arabidopsis* and snapdragon or supercompound organ in tomato. In contrast, reduced miR319 levels or high *TCP* activity reduces leaf and petal size, results in a simple tomato leaf, and is lethal in extreme cases. Insights into the gene networks that are controlled by the miR319-regulated *TCPs* demonstrate their participation in multiple biological pathways, from hormone biosynthesis and signaling to cell proliferation and differentiation.

1 Discovery of miR319 Through a Genetic Screen

MicroRNAs (miRNAs) are a conspicuous group of small RNAs present in animals and plants, which are defined by their unique biogenesis as they are processed from an imperfect fold-back precursor (Meyers et al. 2008). In both plants and animals, miRNAs have been discovered by three methods: direct cloning and sequencing, genetic screenings, and bioinformatic predictions.

The first plant miRNAs were identified by isolating, cloning, and sequencing small RNA populations (Reinhart et al. 2002; Llave et al. 2002; Park et al. 2002). This approach bursts recently with the development of deep-sequencing strategies [e.g., (Fahlgren et al. 2007; Rajagopalan et al. 2006; Lu et al. 2005, 2006)]. Cloning

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and sequencing have led to the discovery of many of the currently known plant miRNAs (miRBASE 17.0, www.mirbase.org).

Several plant miRNAs have also been identified by bioinformatics approaches based on the conservation of the small RNA sequence in the context of a fold-back precursor in different species [e.g., (Jones-Rhoades and Bartel 2004)]. In contrast, very few have been recognized from genetic screenings, probably due to the small size of the small RNA or redundancy with other miRNA-coding genes that have similar or identical sequences.

One exception is miR319a, which was isolated during an activation tagging experiment in which transgenic *Arabidopsis* plants were screened for morphological changes (Weigel et al. 2000). In this type of screen, plants are transformed with a modified T-DNA vector containing a viral enhancer cassette derived from the 35S promoter of the cauliflower mosaic virus. The T-DNA harboring the viral enhancers is integrated randomly into the *Arabidopsis* genome causing the transcriptional activation of nearby genes and generating dominant gain-of-function mutations (Weigel et al. 2000).

One of the recovered mutants that stood out for its unusual morphology was *jaw-D* (Fig. 1). The dominant *jaw-D* phenotype is governed by its pronounced jagged and wavy leaves. Four independent alleles (*jaw-D1* to *jaw-D4*) were isolated from this and other activation tagging screens (Weigel et al. 2000; Palatnik et al. 2003). For all alleles, the insertion site was determined to be on chromosome IV between the protein-coding genes At4g23710 (coding for vacuolar synthase subunit G2) and At4g23720 (coding for a protein of unknown function). Surprisingly, the region responsible for the *jaw-D* phenotype was mapped to 1.6 kb in the intergenic region located between these two genes, where no open reading frame could be found, suggesting that *JAW* might be a nonprotein-coding gene (Weigel et al. 2000).

Microarray experiments comparing wild type with *jaw-D* transcriptomes showed that a group of five *TCP* transcription factors was significantly downregulated in the *jaw-D* mutant. Interestingly, a *TCP* mutant in snapdragon called *cincinnata* had also defects in leaf development (Nath et al. 2003) similar to those described in *jaw-D*, indicating that the decrease in *TCP* levels was underlying the *jaw-D* phenotype. An alignment of these *TCPs* showed that apart from the typical *TCP* domain, they shared a highly conserved short stretch toward the C-terminal part of the protein (Palatnik et al. 2003). Conspicuously, the conservation of this region was found to be exceptionally high at RNA level and the site consisting of 21 nucleotides was found in *TCPs* of about 20 analyzed species, including *CINCINNATA* from snapdragon (Fig. 1).

A short complementary sequence to this conserved motif was identified in the genomic region in the *jaw-D* mutant in proximity to the insertion site of the viral enhancers. The sequence was located at the base of a predicted RNA fold-back structure. Small RNA blots and sequencing of small RNA further demonstrated that a small RNA with the predicted sequence was actually overexpressed in *jaw-D* plants, now known as miR319 (Palatnik et al. 2003). The whole strategy also allowed the identification of miRNA targets by microarrays, an approach that was subsequently used in animals [e.g., (Lim et al. 2005)].

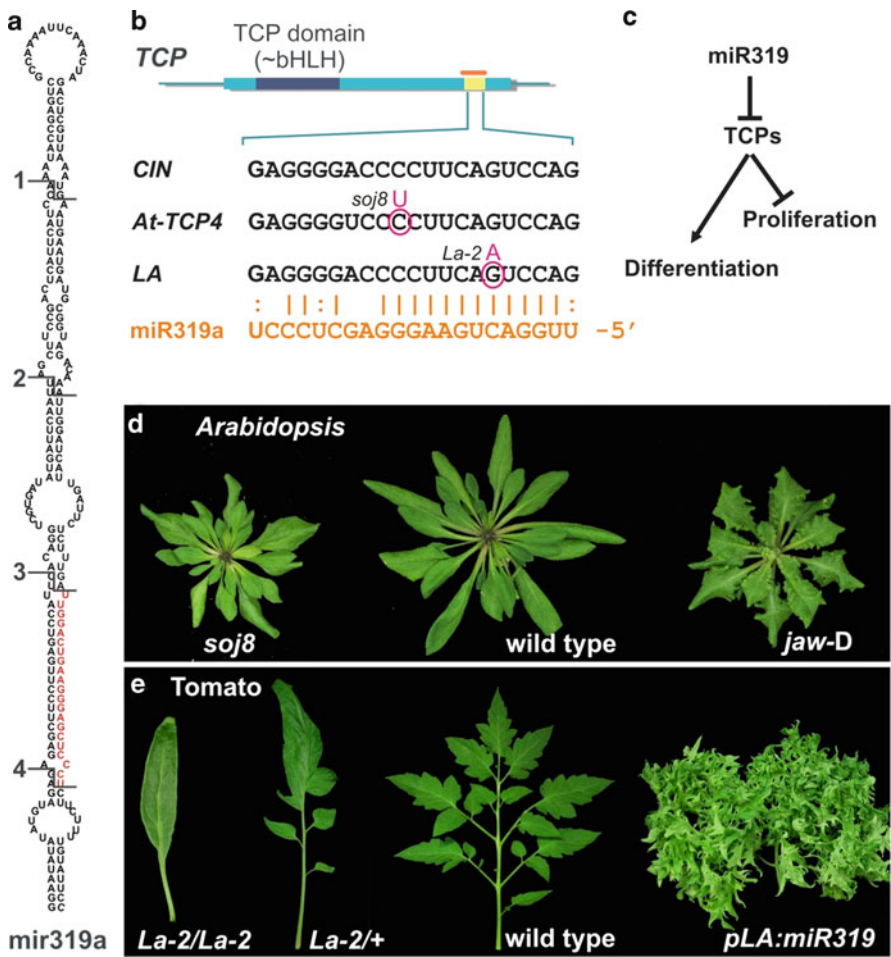


Fig. 1 Biological role of miR319. (a) Secondary structure of the miR319a precursor. The four DICER-LIKE1 cleavage sites required for its processing are indicated by numbers, and the miRNA region is highlighted in orange. (b) Scheme representing a TCP gene. The miRNA target site for *CINCINNATA* (*CIN*) of snapdragon, *Arabidopsis TCP4* (*At-TCP4*), and the tomato *LANCEOLATE* (*LA*) are indicated. The point mutations in *soj8* and *La-2* are shown. (c) Diagram indicating the biological roles of miR319 and the TCPs. (d) *Arabidopsis* plants with different miR319 levels and TCP activity. (e) Tomato leaves with different levels of miR319 and TCP activity

The activation-tagging approach has proven to be powerful to isolate dominant miRNA mutants. For example, the early-flowering *eat-D* mutant was obtained by activation tagging and shown to overexpress miR172b, then called miR172a-2 (Aukerman and Sakai 2003). Furthermore, two family members of the large miR166 family were identified this way, miR166a overexpressors, *meristem enlargement1 D*, and the miR166g overexpressing line, *jabba1-D*, that display leaf defects

and fasciated stems (Williams et al. 2005; Kim et al. 2005). Similar approaches have also been employed in animals and, for example, have allowed the identification of miRNA Bantam in *Drosophila* (Brennecke et al. 2003). Currently, few cases of point mutations in miRNAs have been isolated on the basis of their phenotype. Some exceptions are *mir164c*, affecting the number of petals (Baker et al. 2005), and *mir319a*, which displays modified petal size and shape (Nag et al. 2009).

2 Regulation of TCP Transcription Factors by miR319

The TCPs are a plant-specific transcription factor family; still, the TCP domain codes a motif that is predicted to fold into a basic helix-loop-helix structure known from DNA-binding domains of both plant and animal transcription factors (Cubas et al. 1999). The name TCP is adopted from the founding family members *Teosinte Branched 1* (*TB1*) from maize, the *Antirrhinum* gene *Cycloidea* (*CYC*), and the two PCNA promoter binding factors *PCF1* and *PCF2* from rice [reviewed in (Cubas et al. 1999; Martin-Trillo and Cubas 2010)]. Interestingly, the seminal work in this family of transcription factors has been performed in species different from *Arabidopsis*.

In *Arabidopsis*, the TCPs comprise a family of 24 members which can be subdivided in two main branches (class I and II) according to their sequence in the TCP domain. The *Arabidopsis* TCP transcription factor family has been studied intensely during recent years and has been shown to participate in various important aspects of plant development [recently reviewed in (Martin-Trillo and Cubas 2010)]. TCPs have been involved in different aspects of the control of cell division, expansion, and differentiation during leaf development (Efroni et al. 2008; Koyama et al. 2007, 2010; Sarvepalli and Nath 2011; Nath et al. 2003; Masuda et al. 2008; Schommer et al. 2008; Palatnik et al. 2003; Li et al. 2005a). The functions also include control of branching (Aguilar-Martinez et al. 2007), mitochondrial biogenesis (Gonzalez et al. 2007), leaf senescence (Schommer et al. 2008), flower development (Palatnik et al. 2003; Sarvepalli and Nath 2011; Nag et al. 2009), and male and female gametophyte development (Pagnussat et al. 2005; Takeda et al. 2006). Furthermore, there are data demonstrating an interaction with the circadian clock (Giraud et al. 2010; Pruneda-Paz et al. 2009) and the control of jasmonic acid and auxin biosynthesis and signaling, respectively (Schommer et al. 2008; Koyama et al. 2010).

Of the 24 *Arabidopsis* TCP genes, five contain a target site for miR319: *TCP2* (At4g18390), *TCP3* (At1g53230), *TCP4* (At3g15030), *TCP10* (At2g31070), and *TCP24* (At1g30210). The miRNA target site is in all cases located outside the TCP domain, near the 3' part of the coding region (Fig. 1). All targets of miR319 are closely related members of the class II subclass of TCP genes (Palatnik et al. 2003).

The interaction between the TCPs and miR319 has up to six mismatches, depending on the specific transcription factor considered, which is higher than in other known plant miRNA target pairs (Fig. 1). Still, the predicted free energy is good enough, with ΔG values of -34kcal/mol , to suggest an efficient interaction. It

was confirmed in vivo that *TCP* mRNA fragments, generated by an miR319a-guided cleavage, can be isolated for all of them (Palatnik et al. 2003).

Microarray experiments comparing the transcriptome of wild-type and *jaw-D* plants in the shoot apical meristem showed a clear decrease in the levels of all miR319-targeted *TCP*s, up to 30-fold, which strongly indicates that their RNA was guided to degradation by miR319 activity (Palatnik et al. 2003; Schommer et al. 2008; Efroni et al. 2008). Other *TCP* genes, lacking an miR319 binding site, were largely unaffected. The action mechanism of miR319 on the *TCP*s was further investigated in transient assays in *Nicotiana benthamiana*. Coexpression of miR319 and *TCP4* in *N. benthamiana* leaves led to the complete degradation of the transcription factor RNA (Palatnik et al. 2003).

To study the importance of the regulation of *TCP4* (and other *TCP*s) by miR319, transgenes that avoided the regulation by the miRNA were generated. To this end, silent mutations that abolish the interaction with the miRNA without changing the coding sequence encoded amino acids were introduced into the binding site for miR319 in the *TCP4* (Palatnik et al. 2003; Efroni et al. 2008; Koyama et al. 2007). The resulting *TCP4* transcript was resistant (*rTCP4*) to the presence of miR319 in transient assays (Palatnik et al. 2003).

Expression of transgenic miRNA-resistant *TCP*s in *Arabidopsis* leads to higher levels of mRNA expression and developmental defects (Palatnik et al. 2003; Koyama et al. 2007; Efroni et al. 2008). Mutations in the miRNA target site of *TCP4* (Palatnik et al. 2007) and its homolog in tomato, *LANCEOLATE* (Ori et al. 2007), have also been obtained by EMS mutagenesis and are known to cause an increase in their transcript levels. An accumulation of *TCP* RNA levels was also observed in the *mir319a*¹²⁹ mutant in *Arabidopsis* (Nag et al. 2009). Therefore, current gathered evidence indicates that miR319 regulates the *TCP*s by guiding them to cleavage.

An EMS mutagenesis carried out on the *jaw-D* mutant rendered several suppressors named *soj* (for *suppressor of jaw-D*). Among the suppressors with nearly wild-type leaves, four were mutants with changes in the miR319 binding site of *TCP4* (Palatnik et al. 2007). These mutations partially dampened the interaction with the miRNA, therefore compensating the high levels of miR319 in the *jaw-D* mutant (Fig. 1), further confirming that the *jaw-D* phenotype is caused by the downregulation of the *TCP*s.

3 Functions of miR319-Regulated TCP Transcription Factors

The miR319 regulatory network has been implicated in different aspects of leaf development, from morphogenesis to leaf senescence and from cell proliferation to cell differentiation. These multiple descriptions probably highlight the participation of miR319 and the *TCP*s in many key biological processes. Interestingly, many of the functions of *TCP* transcription factors were first discovered in other species than the model plant *Arabidopsis thaliana*, such as snapdragon or tomato.

3.1 Seedling Development and Embryo Patterning

Stable introduction of an miR319-resistant version of *TCP4* into *Arabidopsis* plants is lethal in most cases (Palatnik et al. 2003; Schommer et al. 2008), while misexpression from tissue-specific promoters at later stages of leaf development can significantly reduce the *Arabidopsis* leaf size (Efroni et al. 2008). Patterning defects, including fused cotyledons, have also been observed in *soj* plants, which have mutations in the miR319 binding site of *TCP4* (Palatnik et al. 2007). At least part of these patterning defects is caused by the repression of *CUC* genes by the TCPs (Koyama et al. 2007).

3.2 Regulation of Leaf Morphogenesis by miR319 and the TCPs

Leaves are determinate organs that have a defined morphology. To acquire their characteristic final size and shape, growth in the developing leaf needs to be tightly coordinated first through cell proliferation and then by cell expansion. Initially, cell proliferation is observed throughout the developing leaf (Donnelly et al. 1999). Cell cycling stops first at the tip of the leaf and then a mitotic arrest front moves toward the base of the organ. Once cells cease to divide, they begin to enlarge, and cell growth becomes the driving force regulating organ size (Tsukaya 2006). The control of these two processes by the developmental program of the plant is responsible for the final shape and size of the leaf generating the multitude of forms that are found in nature.

Normally, snapdragon leaves are flat organs but become crinkled in mutants in the *TCP* gene *CINCINNATA* (Nath et al. 2003). A first analysis revealed that three aspects of leaf morphology were affected in the *cincinnata* leaf: size, shape, and curvature. In situ hybridizations using *HISTONE4* as a marker for cell division showed that the mitotic arrest front moving from the tip to the base of the developing leaf was delayed in a *cincinnata* mutant compared to the wild-type leaf (Nath et al. 2003). Therefore, due to its extended period of growth, especially in the marginal regions, the *cincinnata* leaf would obtain its characteristic crinkled shape (Nath et al. 2003).

Analysis of *CINCINNATA* expression by in situ hybridizations showed that it is expressed in the actively dividing region of the leaf in proximity to the arrest front. For this reason, it was suggested that *CINCINNATA* might be acting in the response of cells to mitotic arrest and be involved in turning off cell proliferation during leaf development (Nath et al. 2003).

In *Arabidopsis*, single knockouts for miR319-regulated *TCP* genes have milder effects on leaf morphology, leading to a slight increase in size (Schommer et al. 2008). However, when double or triple *TCP* knockouts are generated, a crinkled leaf starts to develop, very similar to *jaw-D* (Schommer et al. 2008; Koyama et al. 2010), suggesting that the role of *CIN* in snapdragon is fulfilled by several redundant *TCPs* in *Arabidopsis*.

Interestingly, three additional *Arabidopsis TCP* genes, *TCP5*, *TCP13*, and *TCP17*, seem to play partially redundant functions to the miR319-regulated ones, which collectively are referred to as *CIN-TCPs*, due to their functional and sequence relationship toward the snapdragon *CINCINNATA* (Efroni et al. 2008; Martin-Trillo and Cubas 2010). An artificial miRNA directed against these three *TCPs* increases the crinkled leaf phenotypes of *jaw-D* (Efroni et al. 2008), while mutations in *TCP5* and *TCP13* increase the leaf defects of a *TCP3/4/10* triple mutant (Koyama et al. 2010).

A repressor version of *TCP3* generated by fusing an EAR motif to its coding sequence phenocopies the crinkled leaves of *jaw-D* (Koyama et al. 2007), suggesting that *TCPs* usually fulfill roles as transcriptional activators. Similar results have been obtained with repressor constructs from other *TCP* transcription factors (Koyama et al. 2007, 2010; Shleizer-Burko et al. 2011).

Detailed analysis of leaf development by microarrays has suggested that the miR319-regulated *TCPs* are heterochronic genes that control the progression through different developmental stages (Efroni et al. 2008). Upon lamina initiation, sequential *CIN-TCP* activity promotes the transition from primary morphogenesis to cell expansion and a secondary morphogenesis phase, then regulating cell differentiation in leaves (Efroni et al. 2008).

3.3 Regulation of Leaf Complexity

Tomato leaves are compound organs. However, they become simple organs in the partially dominant *Lanceolate* mutant (Mathan and Jenkins 1960). Recently, several *Lanceolate* alleles were mapped to chromosome seven, allowing the identification of the *LANCEOLATE* gene (Ori et al. 2007). Interestingly, it turned out to be an miR319-regulated *TCP* gene and the mutations mapped to the binding site of the miRNA (Fig. 1) (Ori et al. 2007).

As a consequence of the increased levels of *LANCEOLATE* that escaped the repression by miR319, a simple leaf was generated, which was in turn attributed to a premature differentiation of the leaf (Ori et al. 2007). In contrast, overexpression of miR319 generated a supercompound tomato leaf (Fig. 1). Furthermore, Ori and colleagues detected opposing gradients of miR319 and *LANCEOLATE* expression in developing tomato leaves. While the miRNA was expressed at higher levels in the proximal part of the organ, the *TCP* gene was rather expressed in the distal part.

A further analysis of *Solanaceae* species demonstrated that diverse leaf shapes correlated with different expression patterns of *LANCEOLATE* (Shleizer-Burko et al. 2011). Similar correlations between leaf shape and *LANCEOLATE* expression were observed in different leaves of tomato, whose shape depends on the position on the plant. Moreover, stage-specific expression of miR319 or a repressor version of *LANCEOLATE* generated leaves with different forms (Shleizer-Burko et al. 2011).

The determination of *LANCEOLATE* as an miR319-regulated *TCP* transcription factor and its contribution to the generation of leaves with distinct shape and

complexity highlight the potential role of the miR319 network in the generation of different organ shapes seen in nature. In addition, TCP transcription factors regulate the expression of *CUC* genes in *Arabidopsis*, which are in turn regulated by miR164, a network that has also been implicated in the formation of leaves with different complexity and shapes (see below) (Nikovics et al. 2006; Koyama et al. 2007; Berger et al. 2009; Blein et al. 2008).

3.4 Regulation of Leaf Senescence

Analysis of microarray experiments revealed that genes that are activated by TCPs tend to be expressed at later stages of leaf development, while the ones repressed by these transcription factors are likely to be expressed in younger organs (Schommer et al. 2008; Efroni et al. 2008). The upregulated genes include several genes encoding WRKY transcription factors (Schommer et al. 2008), so named after the first four amino acids of the conserved motif WRKYGQK, which is the hallmark of this family.

One of those genes, *WRKY53*, is an important positive regulator of senescence (Miao et al. 2004; Miao and Zentgraf 2007) which is induced more than 30 times in microRNA-resistant *rTCP4* plants (Schommer et al. 2008). The precocious activation of genes that are normally expressed only during later stages of leaf development in *rTCP4:GFP* is consistent with the role of the snapdragon *TCP* gene *CINCINNATA* as a regulator of the mitotic arrest front during early stages of leaf growth (Nath et al. 2003) and the proposed role for the TCPs in the activation of cell differentiation (Efroni et al. 2008).

Examination of leaf senescence revealed that *jaw-D* leaves had a delay in senescence, while high levels of *TCP4* caused a premature onset of this process (Schommer et al. 2008). The mechanistic pathway that leads to the activation of senescence by the *TCPs* is currently unknown, although there might be interactions with the regulation of jasmonic acid (JA) biosynthesis by these transcription factors (see below), as this hormone has been proposed to be a critical factor in senescence (van der Graaff et al. 2006; Buchanan-Wollaston et al. 2003).

4 Other Functions of miR319 and the TCPs

The section above outlined the importance of the miR319 regulatory network during leaf development. Several studies, however, have revealed that this regulatory node fulfills roles that go beyond leaf development and affect many other processes in the plant.

Firstly, analysis of *cinninata* mutants in *Antirrhinum* showed that in addition to affecting leaf growth, the miRNA-regulated *TCP* gene, *CINCINNATA*, affects petal lobe development by controlling epidermal cell differentiation and growth (Crawford et al. 2004). Furthermore, in a modifier screen in the *dornroeschen-like 2* mutant background, which has flower development phenotypes, an *miR319a*

loss-of-function allele was isolated in *Arabidopsis* (Nag et al. 2009). *mir319a* mutants exhibit defects in petal and stamen development, presenting narrower and shorter petals as well as impaired anther formation (Nag et al. 2009).

Plants with high TCP activity also suffer impaired development of floral organs in *Arabidopsis* (Koyama et al. 2007; Sarvepalli and Nath 2011; Nag et al. 2009) and tomato (Ori et al. 2007). Interestingly enough, petals are greenish in the *jaw-D* mutant, suggesting that a decrease in TCP levels also affects petal development (Palatnik et al. 2003; Weigel et al. 2000).

A large-scale mutant screen of Ds transposon lines allowed the identification of plants defective in early embryo development in which *TCP4* was disrupted (Pagnussat et al. 2005). Furthermore, *jaw-D* mutants have slightly shorter hypocotyls than wild-type plants, whereas increased TCP activity leads to longer hypocotyls (Palatnik et al. 2003; Schommer et al. 2008; Sarvepalli and Nath 2011).

Apart from affecting organ development, the TCPs also have been shown to be involved in the processes of phase change. The *TCP4* mutant and *jaw-D* display a moderate late-flowering phenotype with an increase in leaf number to 22 compared to 15 in wild type (Palatnik et al. 2003; Schommer et al. 2008; Sarvepalli and Nath 2011).

To conclude, microarray profiling for expression changes during *Arabidopsis* photomorphogenesis upon light activation exhibited cotyledon-specific expression of class II miR319-regulated TCPs (López-Juez et al. 2008). In connection to this, a recent study showed that TCPs might be central regulators of the circadian clock, not only by activating the transcription of, but also by directly establishing protein–protein interactions with core components of the clock (Giraud et al. 2010).

5 Gene Networks Controlled by the miR319-Regulated TCPs

Numerous recent studies have begun to tackle a further elusive question: Which are the *in vivo* target genes whose expression is directly governed by miRNA-regulated TCP transcription factors?

5.1 DNA Recognition by TCP Transcription Factors

Early work by Kosugi and Ohashi (2002) made use of random binding site selection (SELEX) and electrophoretic mobility shift assays (EMSAs) to identify the consensus DNA-binding sequences of both class I and II TCP proteins in rice, the PCFs (Kosugi and Ohashi 2002). The sequences identified were GGNCCCAC for class I and GTGGNCCC for class II, which showed a certain degree of overlap.

Also in *Arabidopsis*, efforts were taken to identify the motives to which TCPs bind. An *in silico* approach took as basis genes that were changing expression in microarray experiments with plants of high or low TCP activity. Genes positively

regulated by TCPs were expected to be upregulated in *rTCP* plants, while being downregulated in *jaw-D* or *TCP* knockouts. A list of potential candidates was obtained which, by promoter comparison, allowed the identification an overrepresented motif of a potential TCP binding site, GGACCA (Schommer et al. 2008).

This potential motif was confirmed to be a functional binding site of TCP4 by in vitro SELEX and EMSAs (Schommer et al. 2008). The box was also related to the sequence preferentially bound by rice PCF5, which has an miR319 binding site (Kosugi and Ohashi 2002). More recently, Aggarwal et al. (2010) have pinpointed key residues involved in DNA recognition and dimer formation by TCP4. They found that the TCP domain has binding parameters similar to those of canonical bHLH transcription factors (Aggarwal et al. 2010).

5.2 Direct Targets of miR319-Regulated TCPs

Microarrays of transgenic plants with different levels of miR319 or *TCPs* have been important to identify networks controlled by these transcription factors (Palatnik et al. 2003; Schommer et al. 2008; Efroni et al. 2008). In general, genes induced by *TCPs* tend to be expressed at later stages of wild-type leaf development (Schommer et al. 2008; Efroni et al. 2008).

LIPOXYGENASE2 (*LOX2*) is one of the most affected genes in the transcriptome of *jaw-D* and *rTCP4* plants. It has four TCP binding sites in its promoter and codes for an enzyme of the plant hormone jasmonic acid (JA) biosynthesis pathway (Schommer et al. 2008). Additional enzymes involved in JA biosynthesis also respond to miR319 and *TCP* levels and have TCP binding sites in their promoters (Schommer et al. 2008). The functionality of the TCP binding sites in *LOX2* was tested in vitro and with the aid of GUS reporters *in planta*. In addition, a reduced ability of the *jaw-D* line to produce JA upon wounding was demonstrated which highlights the biological relevance of *LOX2* as a TCP target (Schommer et al. 2008).

The same year, Masuda et al. (2008) unraveled some of the roles of another miR319-regulated TCP transcription factor. By biochemical and genomic approaches, they demonstrated that TCP24 interacted with Armadillo BTB Arabidopsis protein (ABAP1) to negatively regulate the transcription of the pre-replication control (pre-RC) factor genes *CT1a* and *CT1b*, which are required for S-phase entry and DNA replication (Masuda et al. 2008). This dual complex could possibly further regulate cell proliferation by its direct *in vivo* association with other protein components of the pre-RC, such as ORC1a, ORC3, and CT1a, affecting pre-RC assembly and/or origin selection (Masuda et al. 2008).

Chimeric genes expressing a transcriptional repressor domain (SRDX) fused to the TCPs phenocopy the *jaw-D* mutant where miR319 is overexpressed (Koyama et al. 2007). These plants induced the expression of boundary-specific genes from the *CUC* gene family and suppressed the expression of miR164, whose product cleaves the transcripts of *CUC* genes (Koyama et al. 2007).

In a subsequent work, Koyama et al. (2010) used a set of microarray data from plants expressing inducible *TCP* transcription factors to select candidate target genes. Chromatin immunoprecipitation analysis revealed the direct binding of *TCP3* to the promoters of *AS1*, *MIR164a*, and two genes involved in auxin response, *IAA3/SHY2* and *SAUR*. In turn, these genes cooperatively repressed *CUC* genes (Koyama et al. 2010). The authors proposed that *CIN-TCPs* could promote the differentiated fate through *CUC*-dependent and independent pathways and that this functional redundancy would increase the robustness and flexibility of the leaf developmental program (Koyama et al. 2010).

Finally, although no new targets were identified, Giraud et al. (2010) found that miR319-regulated *TCPs* can directly associate with core components of the clock *via* protein-protein interactions. They also found that the abundance of several *TCP* transcripts oscillates in a day/night cycling fashion (Giraud et al. 2010). The potential role of *TCPs* in the regulation of gene expression *via* protein-protein interaction adds an extra layer of complexity to their functions.

6 Interaction Between the miR319 Network and Other miRNAs

6.1 Interaction with the miR164 Network

Recent results indicate that networks of transcription factors regulated by miRNAs can interact with others during plant development [reviewed in (Rubio-Somoza and Weigel 2011)]. Several lines of evidence show a link between miR319 and miR164 regulatory nodes (Koyama et al. 2007, 2010; Hasson et al. 2011; Palatnik et al. 2003).

miRNA miR164 regulates *CUC1* and *CUC2*, as well as other related genes in *Arabidopsis* (Rhoades et al. 2002). These genes belong to the NAC family of plant-specific transcription factors which comprise more than 100 members in *Arabidopsis* (Ooka et al. 2003). *CUC1* and *CUC2* are partially redundant with a third member *CUC3* (Vroemen et al. 2003), which is not an miR164 target. Mutations in two of these genes (Takabe et al. 1997; Takada et al. 2001; Vroemen et al. 2003) or overexpression of the miRNA (Laufs et al. 2004; Mallory et al. 2004) causes a defective shoot apical meristem and cotyledon fusions.

Defects in the *CUC* homologues of petunia (*NO APICAL MERISTEM*), snapdragon (*CUPULIFORMIS*), and tomato (*GOBLET*) lead to similar developmental defects (Souer et al. 1996; Blein et al. 2008; Berger et al. 2009; Weir et al. 2004), revealing an evolutionarily conserved role for *CUC* genes in SAM function and organ separation. More recently, it has been found that these factors are required for leaflet formation in plants with compound organs (Berger et al. 2009; Blein et al. 2008), while the serrations of the *Arabidopsis* simple leaf are regulated by a balance between *CUC2* and miR164a (Nikovics et al. 2006).

Current evidence shows that the miR319 and miR164 regulatory networks interact in several ways. First, a TCP3-SDRX repressor activates *CUC* expression ectopically, and the defects observed in the strongest lines were ameliorated in *CUC1* or *CUC2* mutants (Koyama et al. 2007). On the other hand, it has been suggested that the cotyledon fusions observed in *rTCP4* or *rTCP3* (Koyama et al. 2007; Palatnik et al. 2003) are caused by the repression of *CUC* genes, being *MIR164a* a direct target of TCP3 (Koyama et al. 2010). Interestingly, the crinkles in *jaw-D* leaves are strongly reduced in a *CUC2* mutant background (Hasson et al. 2011).

6.2 Interaction with the miR396 Regulatory Network

miRNA miR396 regulates *GROWTH-REGULATING FACTORS (GRFs)* (Jones-Rhoades and Bartel 2004), which are a plant-specific family of transcription factors (Kim et al. 2003). Overexpression of miR396 causes smaller leaves (Liu et al. 2009; Rodriguez et al. 2010), while an miR396-resistant *GRF2* version increases the organ size compared to wild type (Rodriguez et al. 2010).

MiR396 is expressed during leaf development and accumulates with organ age in an opposing pattern to the *GRFs* and genes involved in the control of the cell cycle (Rodriguez et al. 2010). Ectopic expression of miR396 reduces the expression of *CYCLINB1;1*, *KNOLLE*, and other genes involved in the transition from G2 to M phase of the cell cycle (Rodriguez et al. 2010).

Plants expressing an miR319-resistant version of *TCP4* have increased miR396 levels and a reduction in *GRF* expression. *GRF2* is normally expressed in a proximodistal gradient along the longitudinal axis of a developing leaf. This gradient is reduced and shifted toward the base of the leaf in *rTCP4* transgenics in parallel with a decrease in cell proliferation (Rodriguez et al. 2010). These results suggest that at least part of the effects of *rTCP4* on cell proliferation might be caused by the activation of miR396 and a reduction in *GRF* activity (Rodriguez et al. 2010).

7 The miR319/miR159 Superfamily of Plant miRNAs

Many plant and animal miRNAs are encoded by small gene families. All family members are identical or very similar but are encoded by different genes and precursors. In *Arabidopsis*, the miR319/miR159 family comprises six miRNAs with similar sequences: miR319a, miR319b, miR319c, miR159a, miR159b, and miR159c. All these miRNAs differ in their 3' end, except for miR319a and miR319b, which are identical. The three miR159 miRNAs have two additional differences with respect to miR319: They have a change at position 6 (U for C) and an additional base at their 5' end, but therefore one less base at their 3' end so that miR159 sequences are shifted one nucleotide relative to miR319.

As ARGONAUTE proteins slice their target RNAs at position 10–11 from the 5' end of the miRNA, the products of miR319- or miR159-guided cleavage can then be readily distinguished as they differ in one base. Analysis of *TCP* fragments in vivo revealed that they are created by miR319-directed cleavage. This result is in principle surprising because miR159 is much more abundant than miR319 as judged by small RNA sequencing projects [e.g., (Fahlgren et al. 2007; Rajagopalan et al. 2006; Lu et al. 2006)]. However, site-directed mutagenesis of the *MIR159a* precursor revealed that the differences at position 6 and the 3' end with respect to miR319a ensure that miR159 does not target the *TCPs* (Palatnik et al. 2007).

What are then the functions of miR159 in vivo? Overexpression of miR159 and other studies revealed that miR159 miRNAs target *MYB* transcription factors (Achard et al. 2004; Schwab et al. 2005). As a consequence, the ectopic expression of miR159 does not affect leaf morphogenesis but stamen development (Achard et al. 2004; Schwab et al. 2005; Palatnik et al. 2007). Several of the miR159 targets are grouped as *GAMYB*, genes that were first identified in barley aleurone cells as gibberellin-specific transcriptional regulators (Gubler et al. 1995). Loss-of-function of a rice *GAMYB* affects the gibberellin-mediated induction of the aleurone hydrolyase α -amylase and stamen development in rice (Kaneko et al. 2004). The miR159-regulated *MYB33* and *MYB65* also redundantly regulate stamen development in *Arabidopsis* (Millar and Gubler 2005).

Single knockouts for *MIR159a* and *MIR159b* have been isolated in *Arabidopsis*, and they do not have an obvious phenotype. However, double knockouts have pleiotropic defects, including leaves that are curled upward (Allen et al. 2007). These defects are compensated in *mir159a/mir159b/myb33/myb65* mutants, demonstrating that the ectopic activation of the *GAMYBs* *MYB33* and *MYB65* in the miRNA double mutant is responsible for its developmental problems (Allen et al. 2007). MiR159 in addition regulates other *MYB* transcription factors, such as *DUO1* (Palatnik et al. 2007), although the biological significance of this regulation is unclear (Allen et al. 2010).

In contrast to miR159 that cannot target the *TCPs* due to specific sequence requirements, miR319 can in principle regulate *GAMYBs* (Palatnik et al. 2007). However, the limited expression pattern of miR319 and the abundance of miR159 make miR159 the mayor *GAMYB* in vivo regulator (Palatnik et al. 2007). Due to the fact that miR319 and miR159 have very similar sequences but regulate different genes in vivo, they are sometimes grouped together in one family, while others consider them separately; however, both options are currently accepted (Meyers et al. 2008).

8 Biogenesis of miRNA miR319

Plant miRNA precursors are extremely variable in size and secondary structure, contrasting with their stereotypical animal counterparts (Bologna et al. 2009). Beyond these variations, detailed studies have demonstrated that many plant precursors have a lower stem of ~15 nt below the actual miRNA/miRNA* sequence

(Mateos et al. 2010; Song et al. 2010; Werner et al. 2010). This region is recognized by the processing complex containing DICER-LIKE1 to produce the first cleavage reaction at the base of the precursor (Mateos et al. 2010; Song et al. 2010; Werner et al. 2010).

Interestingly, the precursors of miR319 and miR159 do not contain this lower stem below the miRNA sequence. In contrast, they have long fold-backs with an extended upper stem above the miRNA (Fig. 1). The miR319 miRNAs are widely distributed in plants, including mosses (Arazi et al. 2005; Axtell and Bartel 2005; Axtell et al. 2007), and their large stem-loop sequences are also highly conserved (Palatnik et al. 2003; Li et al. 2005b; Axtell et al. 2007; Warthmann et al. 2008b). Detailed mutagenesis studies and mapping of processing intermediates have demonstrated that miR319 and miR159 precursors are processed by four dicing reactions, instead of the usual two found in other plant and animal miRNAs (Bologna et al. 2009). Most conspicuously, the first cut is produced at the loop, and the processing proceeds in a “loop-to-base” direction, miR319 being released after the third and fourth cuts. This mechanism is also conserved in the moss *Physcomitrella patens*, demonstrating its ancient origin (Bologna et al. 2009; Addo-Quaye et al. 2009).

The fold-back precursor of miR319a has been successfully engineered to express specifically designed small RNA sequences, called artificial miRNAs (Schwab et al. 2006). This approach has been applied in many species, including rice (Warthmann et al. 2008a). It has been suggested that the noncanonical biogenesis of miR319 might contribute to the efficiency of its precursor to express artificial sequences (Addo-Quaye et al. 2009; Bologna et al. 2009).

However, even though there has been a lot of progress in the understanding of the biological function of miR319 and its targets, a group of *TCP* transcription factors, open questions remain. Perhaps the most intriguing one is how miR319 itself is regulated. Future work will likely answer this and other aspects of this key miRNA regulatory network.

Acknowledgments We would like to thank Yogev Burko and Naomi Ori for providing the pictures of *Lanceolate* and miR319 overexpressors in tomato. Our work is supported by grants from the Agencia Nacional de Promoción Científica y Tecnología (to C.S. and J.P.) and the Howard Hughes Medical Institute (J.P.). C.S., S.P., and E.B. are fellows of the Argentinean Research Council (CONICET), and J.P. is member of the same institution.

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MicroRNAs in Plant Development and Stress Responses

Sunkar, R. (Ed.)

2012, VIII, 296 p., Hardcover

ISBN: 978-3-642-27383-4