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DARWIN REVIEW

# Co-ordination of developmental processes by small RNAs during leaf development

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## Abstract

Leaf development entails the transition from a small group of undifferentiated cells to a structure of defined size and shape, highly organized into different cell types with specialized functions. During this developmental sequence, patterning, growth, and differentiation have to be tightly coordinated by intricate regulatory networks in which small RNAs [microRNAs (miRNAs) and *trans*-acting small interfering RNAs (ta-siRNAs)] have emerged during the last years as essential players. In this review, after having given an overview of miRNA and ta-siRNA biogenesis and mode of action, their contribution to the life of a leaf from initiation to senescence is described. MiRNA and ta-siRNA are not merely regulators of gene expression patterns, but, by acting both locally and at the whole organ scale, they have an essential role in the coordination of complex developmental processes and are fully integrated in genetic networks and signalling pathways.

**Key words:** Differentiation, genetic network, growth, leaf, miRNA, morphogenesis, polarity, signalling, ta-siRNA.

## Introduction

The importance of small non-coding RNAs in development was first recognized >15 years ago by the cloning of the *lin-4* locus that controls developmental timing in the worm *Caenorhabditis elegans* (Lee *et al.*, 1993). Today, small RNAs appear as key regulators of a large number of biological processes in most eukaryotes. This diverse group is comprised of small non-protein-coding RNAs, from 20 to 30 nucleotides (nt), which are divided into different subtypes depending on their biogenesis, characteristics, and modes of action. Only two classes of small RNAs, the microRNAs (miRNAs) and the *trans*-acting-small interfering RNAs (ta-siRNAs), have been implicated thus far in plant development. The importance of miRNAs for development is reflected by the dramatic phenotypes resulting from mutations in the miRNA biogenesis/function machinery, phenotypes that explain why these mutants were first identified as developmental mutants before their link with the small RNA pathway was recognized (for a review, see Garcia, 2008). Two examples of this in relation to leaf development are the *argonaute1* mutant, which develops

leaves similar to squid tentacles (Bohmert *et al.*, 1998), and the *serrate* mutant, which has, among other phenotypes, serrated leaves (Prigge and Wagner, 2001). Ta-siRNAs are endogenous siRNAs that, like miRNAs, regulate genes different from those from which they originate and thus act *in trans*. The *TAS3* gene gives rise to ta-siRNAs called ta-siARFs that target *ARF3* and *ARF4* and are the only ta-siRNAs for which a role in plant development is known.

Leaf development is a multifaceted process during which a small group of undifferentiated cells recruited in the meristem will give rise to a flat structure organized into different cell types (for a review, see Tsukaya, 2006). During this developmental sequence, patterning, growth, and differentiation have to be tightly coordinated and intricate regulatory networks involving transcription factors and hormones are at play. This review highlights the essential roles of different small RNAs in these regulatory networks, but first some general background of miRNA and ta-siRNA biogenesis, action, and regulation is provided.

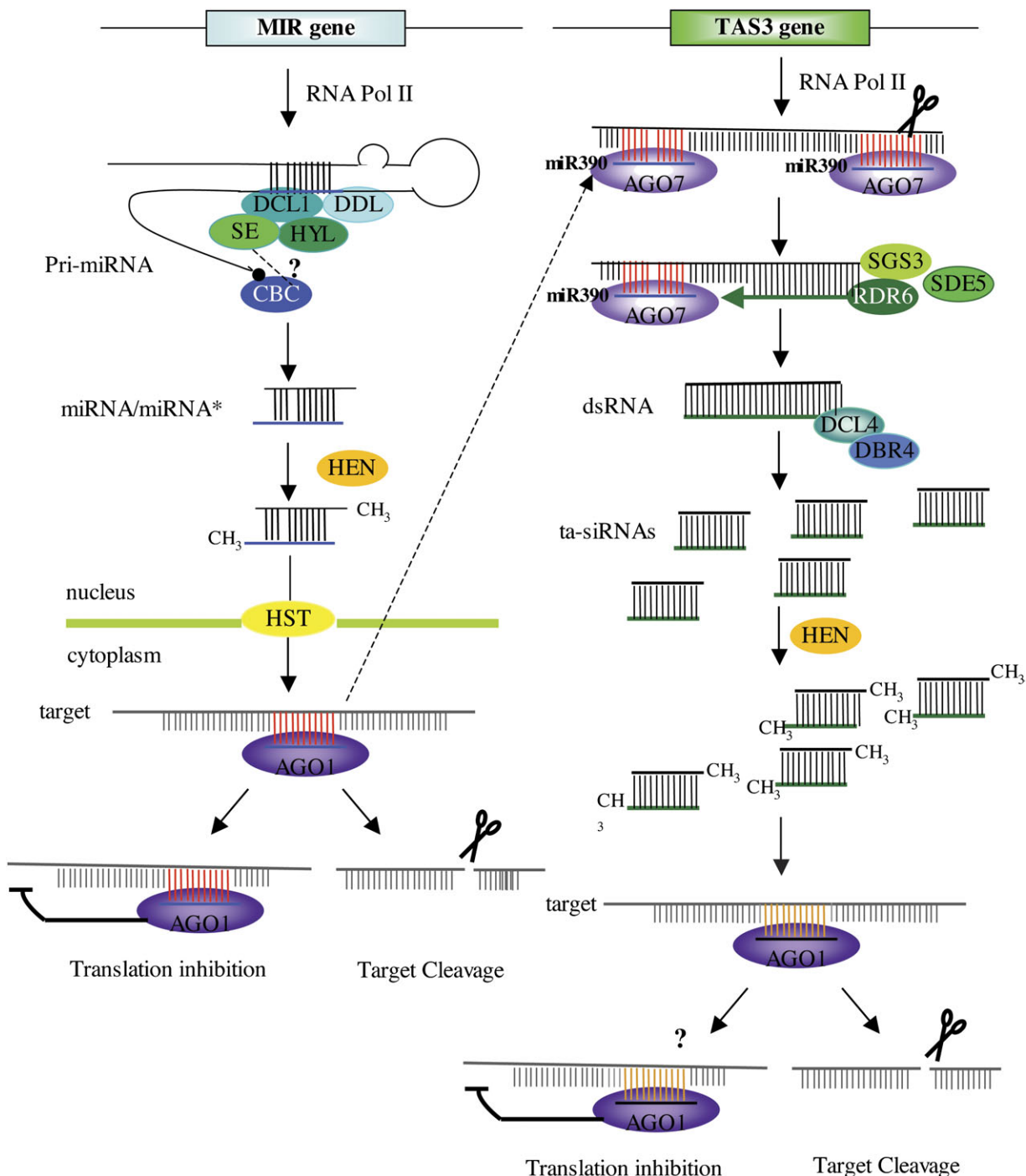
### Overview of miRNA and ta-siRNA biogenesis and action

*How are miRNAs and ta-siRNAs produced?*

Although both miRNAs and ta-siRNAs act as negative regulators by repressing mRNA targets containing a complementary binding site, they originate through distinct pathways and appear to have some differences in their

mechanism of action (Fig. 1, for recent reviews on small RNA production and action see, for example, Willmann and Poethig, 2007; Garcia, 2008; Mallory and Bouche, 2008; Mallory *et al.*, 2008; Ramachandran and Chen, 2008; Voinnet, 2009).

MiRNAs are produced from larger RNA precursors (pri-miRNAs), often from independent transcriptional units, that contain a self-complementary fold back structure



**Fig. 1.** Model for plant miRNA and ta-siRNA ARF biogenesis. MiRNAs are produced by the maturation of long precursors and bind to target genes, which they regulate by cleavage and/or inhibition of translation. The miRNA pathway is also required for ta-siRNA synthesis. In the case of the ta-siARFs, the TAS3 transcripts are doubly targeted by the complex miR390-AGO7. See text for further details.

(Fig. 1). From the RNA precursor, a 21 nt miRNA–miRNA\* duplex is liberated by the coordinated action of the ribonuclease DCL1 (DICER-LIKE 1) (Kurihara and Watanabe, 2004) and its interacting partners: the dsRNA-binding protein HYL1 (HYPOPLASTIC LEAVES 1) (Han *et al.*, 2004), DDL (DAWDLE), which probably facilitates RNA precursor recognition (Yu *et al.*, 2008), and the zinc-finger protein SE (SERRATE) (Lobbes *et al.*, 2006; Yang *et al.*, 2006). Two cap-binding complex proteins CBP20 and ABH1/CBP80 are also involved in this process (Laubinger *et al.*, 2008). To protect the miRNA–miRNA\* duplex from degradation, it is 3' methylated by the methyltransferase HEN1 (HUA ENHANCER 1) (J Li *et al.*, 2005), and exported to the cytoplasm by HST (HASTY) (Park *et al.*, 2005), although it is not yet known which of these two steps occurs first. Upon export, the two strands of the miRNA duplex are separated and a single-stranded miRNA is loaded in the RNA-induced silencing complex (RISC), always in association with an ARGONAUTE PROTEIN, which for most of the miRNAs is AGO1 (for a review, see Vaucheret, 2008). The RISC complex then guides cleavage and/or translational repression of the target mRNAs (Baumberger and Baulcombe, 2005; Qi *et al.*, 2005).

Ta-siRNA biogenesis depends on both the miRNA pathway described above and elements of the siRNA pathway (Fig. 1). Ta-siRNAs derive from long transcripts of *TAS* genes, which contain specific miRNA-binding sites (Peragine *et al.*, 2004; Vazquez *et al.*, 2004; Allen *et al.*, 2005). In the case of *TAS3*, ta-siRNAs originate from the double targeting of the *TAS3* transcript by miR390 complexed with AGO7 (Axtell *et al.*, 2006; Montgomery *et al.*, 2008). In *Arabidopsis*, the miR390–AGO7 complex induces cleavage at the 3' miR390-binding site but not at the 5' site due to mismatches. After *TAS3* precursor cleavage, SGS3 (SUPPRESSOR OF GENE SILENCING 3) stabilizes the 5' cleavage product and recruits RDR6 (RNA DEPENDENT RNA POLYMERASE 6), which catalyses the polymerization of a second RNA strand extending to the 5' miR390-binding site. Next, DCL4 (DICER-LIKE4) (Gascioli *et al.*, 2005; Xie *et al.*, 2005) and its interacting partner DRB4 (ds-RNA BINDING protein 4) (Nakazawa *et al.*, 2007) process the dsRNA to generate a population of 21 nt ta-siRNAs that are in phase with the miR390 cleavage site. Thus, the register of the ta-siRNAs, and hence their sequence and subsequently their targets, are determined by the initial cleavage site of the miRNA. The ta-siRNAs are methylated by HEN1 and incorporated in the RISC containing AGO1 where they direct cleavage of complementary mRNAs.

In comparison with miRNA production, ta-siRNA biogenesis requires a much larger set of components, including miRNA and siRNA pathway components. Differences between ta-siRNA and miRNA activity have been suggested recently in both maize (Chitwood *et al.*, 2009) and *Arabidopsis thaliana* (Schwab *et al.*, 2009). Whereas miRNAs are essentially not or only weakly mobile, ta-siRNAs may diffuse over a larger distance and act in domains different from those where they are produced. These differ-

ences may help to explain the co-existence of these two different types of small RNAs.

#### *What are the effects of miRNAs and ta-siRNAs on target expression?*

Plant miRNAs have a higher base pairing requirement compared with animal miRNA, which implies that plant miRNAs have a more limited number of targets. mRNA cleavage had long been considered the predominant mechanism used by plant miRNAs, but evidence is building for translational inhibition by plant miRNA and other siRNAs (Aukerman and Sakai, 2003; Chen, 2004; Gandikota *et al.*, 2007; Brodersen *et al.*, 2008; Dugas and Bartel, 2008; Lanet *et al.*, 2009).

MiRNA regulation can have different regulatory roles on target gene expression patterns and levels. MiRNAs can contribute to delimitate either the spatial or temporal accumulation patterns of the targets. In addition, miRNAs may have a buffering role to avoid minor changes in target expression levels. Interestingly, the same miRNA may regulate its targets in distinct ways, depending on the species (see miR164 example below).

#### *How is the expression of miRNAs and ta-siRNAs regulated?*

Since miRNAs and ta-siRNAs carry out important regulatory functions they have to be finely and precisely modulated. First, there is a self-feedback regulation to ensure adequate overall miRNA levels. For example, different elements of the miRNA biogenesis machinery, such as DCL1 and AGO1, are themselves regulated by miRNAs (Xie *et al.*, 2003; Vaucheret *et al.*, 2006). In many cases, a single miRNA sequence can be produced from one of several *MIRNA* genes (e.g. miR164 has the potential to be made from three genes, *MIR164a*, *MIR164b*, and *MIR164c*), opening the door to diverse transcriptional regulation. Indeed, at the transcriptional level, *MIRNA* promoters present tissue-specific elements and/or binding sites for different transcription factors, which are in turn related to hormone signalling. This regulation contributes to determine the spatial expression pattern of the mature miRNAs. Recently, it has been suggested that histone acetylation may also regulate miRNA expression at different levels (W Kim *et al.*, 2009). Altogether, this indicates that miRNA genes, being transcribed by the RNA polymerase II complexes, are subjected to the same kind of regulatory mechanisms as protein-encoding genes.

#### *How were the miRNAs and ta-siRNAs and their targets identified?*

Plant miRNAs were identified by sequencing of small RNA libraries, which benefited greatly from the improvement of high-throughput, large-scale sequencing technologies. In parallel, *in silico* pipelines were developed for the prediction of *MIRNA* genes based on the available genomic data. MiRNA targets were predicted based on their extensive



complementarity with the small RNA sequence, and were experimentally validated by 5' RACE (rapid amplification of cDNA ends) analysis of transcript cleavage and transcriptome analysis of miRNA-overexpressing lines. Altogether, these studies have revealed a population of miRNAs and targets conserved throughout land plants. These miRNAs often accumulate to high levels and usually direct repression of genes encoding developmental regulators, whereas a second population of non-conserved miRNAs generally accumulate to lower levels and have more diverse targets.

The importance of regulation by miRNAs has been recognized through forward genetic screens for developmental mutants. For instance, the floral defects in the *Arabidopsis early extra petals1* mutant are due to the mutation of an miRNA gene (Baker *et al.*, 2005), and the leaf polarity defects in the *phabulosa* mutant are due to mutations in an miRNA-binding site (Mallory *et al.*, 2004b). However, most of our knowledge about the functional importance of miRNA regulation stems from reverse genetic approaches including expression of miRNA-resistant targets (in which the miRNA-binding site harbours silent mutations), overexpression and knock-outs of *MIRNA* genes, and inhibition of miRNA activity by the expression of a non-cleavable target mimic RNA that traps the miRNA (Franco-Zorrilla *et al.*, 2007). These strategies and others were used to study the roles of small RNAs during the development of leaves.

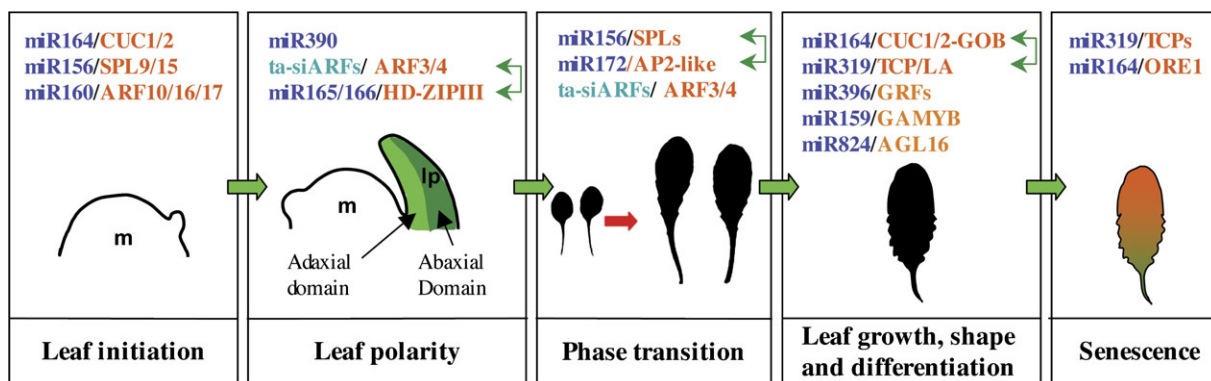
### MiRNAs regulate timing and spacing during early stages of leaf development.

Leaves, which show a regular pattern along the stem called phyllotaxis, originate from the SAM (shoot apical meristem). This small group of dividing, undifferentiated cells is not only the ultimate source of the entire shoot but also controls the positioning and timing of organ initiation (for reviews, see Piazza *et al.*, 2005; Carraro *et al.*, 2006). The site of organ initiation is determined by local peaks of auxin response (for a review, see Veit, 2009). Auxin is actively

transported in the meristem by a complex set of influx and efflux transporters that are often asymmetrically distributed within the cells. The dynamic of the resulting auxin gradients has been proposed to be the basis of the regular pattern of organogenesis in the meristem. However, it is not clear whether the timing of organ initiation is merely a consequence of this system or if it is controlled independently by a clock-like mechanism. MiRNAs are involved in the regulation of both position and time of organ primordia emergence and in their very early development (Fig. 2).

MiR156 regulates plastochron length (the time between the initiation of two successive leaves, the inverse of the leaf initiation rate) by quantitatively modulating the levels of SPL (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE) transcription factors (Schwarz *et al.*, 2008; Wang *et al.*, 2008). Overexpression of miR156 accelerates the rate of leaf initiation. A similar effect is observed in the *sp19 sp15* double mutant, indicating that within the 11 *SPL* genes that are targeted by miR156 in *Arabidopsis*, the regulation of *SPL9* and *SPL15* is the most relevant for the control of plastochron length. *SPL9* mRNA is detected in the developing leaves and is absent from the meristem (Wang *et al.*, 2008). Although miR156 is ubiquitously expressed in the meristem and leaves, it does not appear to contribute to *SPL9* mRNA exclusion from the meristem but controls the *SPL9* level in the leaves. Modulation of *SPL9* expression specifically in the leaves, via the localized expression of miR156 or an miR156-resistant *SPL9* form, modifies the leaf initiation rate in the meristem. This result indicates that a leaf-derived signal, which is sensitive to *SPL9* activity levels, inhibits organ initiation in the meristem. The inhibitory mobile signal could well be the *SPL* proteins themselves or a secondary signal directly regulated by the *SPL*s. In particular, *SPL* proteins could modulate auxin accumulation or response.

Several miRNAs target components involved in auxin reception or response, such as some of the *ARF* (*AUXIN RESPONSE FACTORS*) genes, which code for a large family of transcription factors that mediate many of the auxin responses (for a review, see Rubio-Somoza *et al.*,



**Fig. 2.** Small RNAs and their targets regulate the different stages of leaf development from initiation to senescence. MiRNAs and ta-siRNAs are in dark blue and light blue, respectively, and their targets in orange. Interactions between small RNA/target regulatory modules are shown by green double arrows. m, meristem; lp, leaf primordium.

2009). In relation to leaf initiation, miR160 targets *ARF10*, *ARF16*, and *ARF17*, three members of a divergent class of *ARF* genes that share high amino acid sequence similarity and present overlapping expression patterns. In different organs during plant development, these ARFs carry out specific and overlapping functions, which are all regulated by miR160 (Mallory *et al.*, 2005; Wang *et al.*, 2005; Liu *et al.*, 2007). MiR160 regulation of these *ARF* genes is necessary for proper phyllotaxis in the rosette. In transgenic plants expressing an miR160-resistant form of *ARF10* or *ARF17*, cotyledon numbers and positions are often abnormal and the first three or four true leaves frequently emerge at the same time. Leaves also are often up-curved, strongly serrated, and asymmetric. The expression of different auxin early response genes also increases in these lines, suggesting that this regulatory module contributes to the regulation of auxin response. Both miR156 and miR160 are conserved from mosses to higher plants, suggesting that they regulate ancient and possibly conserved mechanisms in land plants (Floyd and Bowman, 2007).

Local peaks of auxin response in the meristem lead to the definition of the lateral organ founder cells, which are marked by the repression of the *KNOXI* meristematic genes (for reviews, see Carraro *et al.*, 2006; Scofield and Murray, 2006; Hay and Tsiantis, 2009). These cells proliferate faster than the rest of the SAM, and cell division is accompanied by cell expansion. A boundary domain, in which cell expansion is reduced, is established around the primordium to separate it from the neighbouring tissues (for reviews, see Aida and Tasaka, 2006; Rast and Simon, 2008). MiR164 regulates organ boundary size by its modulation of the *CUC1* (*CUP-SHAPED COTYLEDON1*) and *CUC2* genes (Laufs *et al.*, 2004; Mallory *et al.*, 2004a; Baker *et al.*, 2005; Sieber *et al.*, 2007). The *CUC1* and *CUC2* genes belong to the NAC-domain transcription factor family, which has >100 members in *Arabidopsis*. MiR164 has six confirmed NAC targets: *NAC1*, *CUC1*, *CUC2*, *At5g07680*, *At5g61430*, and *ORE1/NAC2*, and one predicted target, *At3g12977*. *CUC1*, *CUC2*, and *CUC3* (which does not possess the miR164 target site) are expressed in the boundary domain around the primordia, and, by repressing growth of these cells, allow organ separation. Single mutants in any of these genes show only mild organ fusion defects, a defect that becomes much more pronounced when two *CUC* genes are inactivated simultaneously, revealing a partial functional redundancy. Plants overexpressing miR164 present the same organ fusion defects as a *cuc1 cuc2* double mutant, whereas, conversely, an miR164-resistant *CUC2* version causes an enlargement of the boundary domains (Laufs *et al.*, 2004). Expression of an miR164-resistant *CUC1* gene leads to the formation of extra petals, a phenotype that is also observed in the *early extra petals1* mutant affected in the *MIR164c* gene, indicating that boundary homeostasis is required for proper organ initiation (Mallory *et al.*, 2004a; Baker *et al.*, 2005). Proper *CUC2* regulation by miR164 is also important during stem growth to maintain the phyllotaxis set-up in the meristem (Peaucelle *et al.*, 2007). The three *Arabidopsis* *MIR164* genes redundantly contribute to

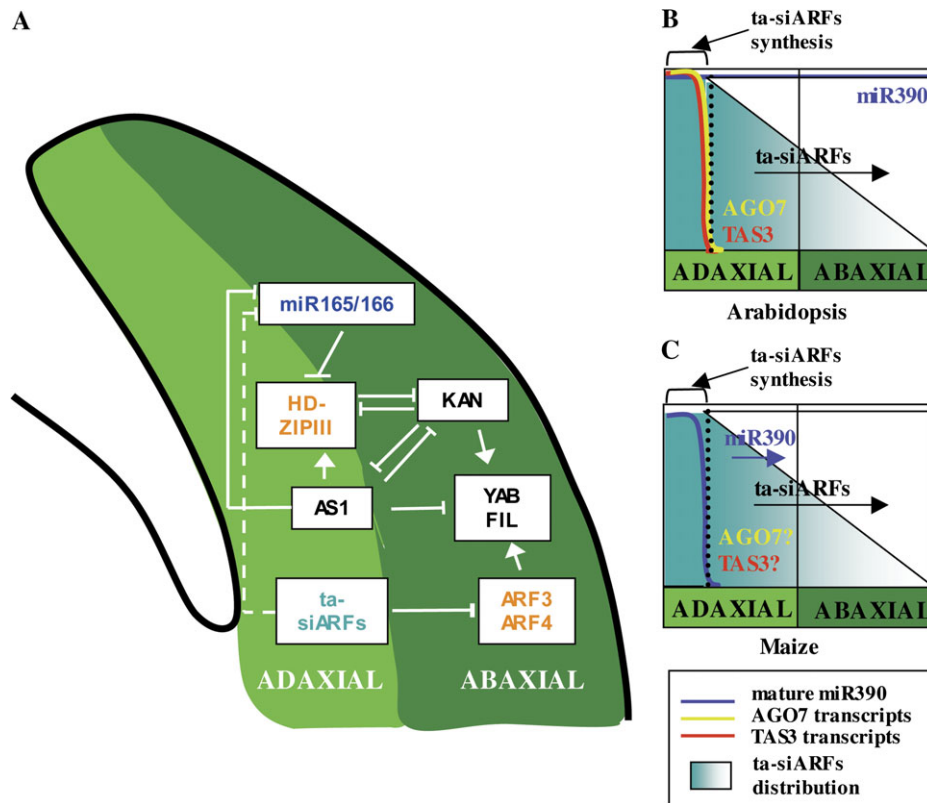
*CUC2* regulation during stem growth, as phyllotaxis defects similar to those of a *CUC2*-miR164-resistant plant are only observed in the *mir164a mir164b mir164c* triple mutant (Sieber *et al.*, 2007). The miR164/*CUC* regulatory unit is found in both dicots and monocots (Floyd and Bowman, 2007).

## Two classes of small RNAs interact to control leaf polarity

Early in leaf development, three main axes (proximo-distal, medio-lateral, and adaxial-abaxial) are established, and these axes need to be maintained throughout the developmental process (for a review, see Byrne, 2005). The presence of the leaf petiole and the lamina reflects the existence of a proximo-distal axis, whereas the midvein and leaf margins define the medio-lateral polarity. Finally, the adaxial-abaxial polarity is reflected in the two opposing sides of the leaf blade, which possess cells with different functions: cells of the adaxial (upper) part being specialized in photosynthesis and cells of the abaxial (lower) part optimized in gas exchange. This specialization is one of the main adaptations of land plants since it maximizes photosynthesis with a minimum water loss to the environment.

The establishment of the adaxial-abaxial polarity occurs early in leaf development and is concomitant with primordium outgrowth. Classic surgical experiments showed that a positional signal emanating from the meristem is required to specify adaxial fate (Sussex, 1954), but this signal has not yet been identified. It is, however, currently clear that abaxial-adaxial polarity is maintained through the antagonistic relationships between two groups of polarity regulators that determine adaxial and abaxial identities, respectively (Figs 2, 3A; for review, see Husbands *et al.*, 2009). The HD-ZIPIII (class III HOMEODOMAIN LEUCINE ZIPPER) and the MYB protein AS1 (PHANTASTICA/ASYMMETRIC LEAF) are adaxial determinants, whereas KANADI (KAN) and the ARF3/ETT, ARF4 proteins promote abaxial fate. The *YABBY* genes act downstream of the *KAN* genes in *Arabidopsis* to specify abaxial fate. This regulatory network is reinforced by the actions of two types of small RNAs, the miRNAs miR165/miR166 and the ta-siRNAs ta-siARFs.

The *Arabidopsis* *HD-ZIPIII* family includes five members that have multiple, redundant, interdependent, and sometimes competitive roles in plant development (Prigge *et al.*, 2005). Three of these genes have been implicated in the definition of the adaxial leaf domain: *PHB* (*PHABULOSA*), *PHV* (*PHAVULOTA*), and *REV* (*REVOLUTA*) (McConnell *et al.*, 2001). All the *HD-ZIPIII* members are targeted by the miR165/miR166 group (two related miRNAs that only differ in sequence by 1 nt), and this regulation is conserved from higher plants to the mosses (Floyd and Bowman, 2004). MiR165/miR166 are encoded by multiple genes (two and seven, respectively, in *Arabidopsis*), but laser-assisted microdissection revealed that only a subset of these genes actually contributes to the



**Fig. 3.** Small RNAs and the establishment of adaxial–abaxial polarity. (A) Schematic representation of the antagonistic interactions between the adaxial (*HD-ZIPIII*, *ARP*, and the small RNAs ta-siARFs) and abaxial determinants (*YAB*, *KAN*, *ARF3/ARF4* and the small RNAs miR165/miR166), which establish a robust regulatory network. (B and C) The gradient of ta-siARFs along the adaxial–abaxial axis is established in different ways in *Arabidopsis* and maize. (B) In *Arabidopsis*, *TAS3* gene transcripts and *AGO7* mRNA, both necessary for ta-siARF production, are restricted to the few most adaxial cell layers of the leaf primordium, restricting ta-siARF biogenesis to this area. Ta-siARFs then form a gradient from this region across the leaf, which is responsible for the polarized accumulation of the ARFs in the abaxial domain. (C) In maize, miR390, the miRNA necessary for the synthesis of ta-siARFs, accumulates adaxially and restricts ta-siARF production to this area. The localization of *TAS3* and *AGO7* transcripts is unknown. As in the case of *Arabidopsis*, from this area a gradient of ta-siARF is established across the leaf.

establishment of maize leaf polarity, suggesting subfunctionalization of the *MIRNA* genes (Nogueira *et al.*, 2009).

The role of the *PHB* and *PHV* genes in leaf polarity was first recognized via the identification of semi-dominant alleles that led to leaf adaxialization (McConnell *et al.*, 2001). Because all these mutations are affected in a putative lipid-interacting domain, it was proposed that the mutant proteins could show an abnormal response to a putative lipidic polarizing signal produced by the meristem (McConnell *et al.*, 2001). The recognition that these mutations also disrupted the miR165/miR166-binding site, and that similar polarity phenotypes resulted from the expression of *PHB* or *REV* genes harbouring silent mutations in the miRNA-binding site indicated that the polarity defects were due to reduced miRNA efficiency (Rhoades *et al.*, 2002; Emery *et al.*, 2003; Mallory *et al.*, 2004b). In these polarity mutants, the expression of the *HD-ZIPIII* gene is not restricted to the adaxial domain, as in wild-type plants, but extends to the entire primordium, indicating that miR165/miR166 targeting is required for spatial restriction of *HD-ZIPIII* expression (McConnell *et al.*, 2001; Juarez *et al.*, 2004; Kidner and Martienssen, 2004). In maize, miR166 accumulates most

abundantly immediately below the incipient leaf, and a gradient of miR166 extends into the abaxial side of the initiating organ generating a pattern complementary to its targets (Juarez *et al.*, 2004). Whether the same also occurs in *Arabidopsis* is not so clear: whereas Kidner *et al.* (2004) reported complementary expression patterns between the adaxial target and the abaxially restricted miRNA, H Li *et al.* (2005) observed miR165/166 expression throughout the leaf primordia.

The ta-siARFs, which are derived from the *TAS3* genes (Adenot *et al.*, 2006; Fahlgrén *et al.*, 2006; Garcia *et al.*, 2006; Hunter *et al.*, 2006), are involved in adaxial determination by restricting the expression of the *ARF3* and *ARF4* genes to the abaxial domain. Ta-siARFs accumulate in a gradient, with higher levels in the adaxial domain of the leaf in both maize and *Arabidopsis* (Chitwood *et al.*, 2009; Nogueira *et al.*, 2009; Schwab *et al.*, 2009). This gradient is a consequence of the adaxially localized production of ta-siARFs and their movement within the primordium (Fig. 3B,C). The mechanisms at the basis of localized production of ta-siARFs are different between *Arabidopsis* and maize. In *Arabidopsis*, *AGO7* and *TAS3* expression, which is required



for ta-siARF production, is restricted to the epidermal and subepidermal layers of the adaxial primordium domain, whereas miR390 is detected throughout the primordium (Fig. 3B). In contrast, localized ta-siARF production in maize relies on *MIR390* polarized expression in the adaxial domain (Fig. 3C). Interestingly, in maize, whereas mature miR390 is detected over a few cells in the adaxial domain, its precursor is restricted to the epidermal layer, indicating that miR390 may diffuse over a few cells. Whether the ability of miRNAs to move over a few cells is a general characteristic of miRNAs or may be specific to this miRNA due, for instance, to its association with AGO7 when other miRNAs are associated with AGO1 is still under debate.

Whereas both numeric simulations and experimental evidence suggest that gradients of ta-siARFs may contribute to sharpen the expression patterns of their targets *ARF3/ETT* and *ARF4* (Levine *et al.*, 2007; Chitwood *et al.*, 2009; Schwab *et al.*, 2009), their importance in leaf polarity may depend on the species. Interfering with ta-siARFs has a clear effect on maize leaf polarity (Nogueira *et al.*, 2007), while this pathway controls *Arabidopsis* leaf polarity redundantly with other factors (Garcia *et al.*, 2006). In both species, however, the adaxial ta-siARF factors are interconnected with abaxial determinants. In particular, a functional ta-siRNA pathway is required to repress the expression of miR165/miR166 (H Li *et al.*, 2005; Nogueira *et al.*, 2007).

Altogether, the evidence presented above underlines the complexity that can exist in small RNA production, movement, and action during leaf polarity.

## Local and global growth control by miRNAs during leaf shaping

Leaf development proceeds through different steps during which cell proliferation, cell expansion, and cell differentiation occur (for a review, see Tsukaya, 2006). Proper leaf development requires a tight coordination of these processes in time and space, both locally, to generate the marginal dissections such as serrations or leaflets, and at the whole organ scale to achieve a flat structure of proper size. Several miRNAs have emerged as regulators of key events in leaf shaping and growth.

Variation in leaf shape results in large part from differences in their level and pattern of dissection: entire leaves are formed by a single unit whereas compound leaves are formed by several independent units called leaflets. The leaf or leaflet margin can be smooth (entire) or further dissected (lobed or serrated). Serrations, lobes, and leaflets are initiated from the primordium margin, a region sometimes called the blastozone (Hagemmann and Gleissberg, 1996), and recent evidence indicates that conserved regulatory factors, including two miRNAs, control the development of these structures (for reviews, see Champagne and Sinha, 2004; Blein *et al.*, 2009).

Local peaks of auxin response create the pattern of the marginal outgrowth in both simple and compound leaves

(Hay *et al.*, 2006; Barkoulas *et al.*, 2008; Koenig *et al.*, 2009). Expression of the *CUC* genes in the boundaries of the outgrowing marginal structures is required for their formation and separation: inactivation of these genes leads to smooth margins, and fused and fewer leaflets (Nikovics *et al.*, 2006; Blein *et al.*, 2008; Berger *et al.*, 2009). The balance between miR164 and *CUC2* determines the level of serration of the *Arabidopsis* leaf: the stronger the *CUC2* activity, the deeper the serrations (Nikovics *et al.*, 2006). Interestingly, although three genes contribute to miR164 production in *Arabidopsis*, *MIR164a*, which has an expression pattern overlapping with that of its target *CUC2* in the sinus of the serrations, has a prevalent role in directing leaf margin development. Beside this, miR164 regulation of *CUC1* is also necessary for proper leaf development: expression of an miR164-resistant *CUC1* gene leads to reduced rosette leaf petioles and deformed leaves (Mallory *et al.*, 2004a). How this relates to miR164 regulation of *CUC1* during normal development remains unclear.

MiR164 is also involved in tomato leaflet initiation by targeting the *CUC*-like gene *GOB* (*GOBLET*). Yet, in contrast to miR164/*CUC2* overlapping expression patterns at the *Arabidopsis* leaf margins, miR164 and *GOB* show complementary expression patterns during tomato leaflet initiation (Berger *et al.*, 2009). When miR164 regulation of *GOB* is disrupted, leaf maturation is delayed and cells remain undifferentiated for a longer period of time. Higher order leaflets are initiated more rapidly and fail to separate properly. This leads to an apparently simpler leaf, which does not result from reduced leaflet formation but from defects in their separation. miR164 is, therefore, essential for a time-regulated and a spatially restricted expression of the *GOB* gene, which in turn is required for a normal leaflet initiation pattern. As the regulation of *CUC* genes by miR164 is conserved in Angiosperms, it would be interesting to explore its role in species with contrasted leaf architectures.

The miRNA miR319, also called miR<sub>JAW</sub> in *Arabidopsis*, is involved in the coordination of cell division and growth during leaf development by targeting a subset of the *TCP* (*TEOSINTE BRANCHED/CYCLOIDEA/PROLIFERATING CELL FACTOR*) genes that are homologues to the *Antirrhinum CIN* (*CINCINNATA*) gene (Nath *et al.*, 2003; Palatnik *et al.*, 2003) and the tomato *LA* (*LANCEOLATE*) gene (Ori *et al.*, 2007). *TCP* proteins play important roles in regulating different aspects of plant shape and form (Cubas *et al.*, 1999). They synchronize cell division and growth, probably by the regulation of genes involved in the cell cycle and ribosomal machinery (C Li *et al.*, 2005).

*Antirrhinum cin* mutants display defects in leaf shape, size, and curvature: their laminas are larger, rounder, and crinkled compared with the wild type (Nath *et al.*, 2003). During wild-type leaf development, a front of cell cycle arrest moves gradually from the tip to the base following a weakly convex pattern. In *cin* mutants, however, the arrest front progresses more slowly and is strongly concave. As a result, cells proliferate longer at the margin of the *cin* mutant, thus leading to the leaf phenotype. As *CIN* is expressed proximal to the arrest front, it was proposed that



it might make cells more sensitive to the cell cycle arrest signal (Nath *et al.*, 2003).

In *Arabidopsis*, plants overexpressing miR319/miRJAW share the same leaf developmental defects as *cin* mutants, but also show higher marginal dissection. The leaf phenotype is due to the multiple targeting of *TCP* genes by the miRNA, in particular *TCP2*, *TCP4*, and *TCPI0* (Palatnik *et al.*, 2003, 2007; Schommer *et al.*, 2008). Conversely, nearly all lines expressing a *TCP4* gene resistant to miRJAW were arrested at the seedling stage and showed phenotypes resembling those of auxin inhibitors (Palatnik *et al.*, 2003), hinting at a possible link with auxin signalling (Rubio-Somoza *et al.*, 2009).

In tomato, miR319 targets the *LA* *TCP* gene and has a major role in the definition of the spatial and temporal competence window during which leaflets originate (Ori *et al.*, 2007). Mutants heterozygous for the semi-dominant *la* mutation display leaves with fewer leaflets and homozygous mutants form simple leaves. *la* mutants are *LA* gain-of-function mutants that present a reduced sensitivity to miR319 due to mutations in the miR319-binding site. In the wild type, miR319 is expressed in the meristem and in young developing leaves where it maintains *LA* expression at a low level without removing it completely. In the mutants, higher *LA* levels are observed in these tissues, which lead to precocious differentiation and an inability to initiate leaflets or to maintain the meristem. On the other hand, increased miR319 expression delays differentiation, which leads to the formation of higher order leaflets and to a super-compound tomato leaf. Altogether, studies in tomato, *Arabidopsis*, and *Antirrhinum* indicate that cell differentiation, and hence growth potential, is fine-tuned by the miR319–*TCP* interaction (Nath *et al.*, 2003; Palatnik *et al.*, 2003; Ori *et al.*, 2007). Increasing this potential leads to the reiteration of the leaf marginal dissection pattern specific to each species: formation of extra leaflets in compound leaves (Ori *et al.*, 2007), increased serrations in simple serrated leaves (Palatnik *et al.*, 2003), and only leaf rumpling with no *de novo* appearance of dissection in the case of entire leaves (Nath *et al.*, 2003). Although miR319/miRJAW is present in Gymnosperms, it appears to regulate genes other than *TCP* genes and its role remains unknown (Axtell and Bartel, 2005; Lu *et al.*, 2007).

When the *TCP* activity is reduced as a result of miR319/miRJAW overexpression, the level of marginal dissection is increased. In *Arabidopsis*, a similar phenotype is observed when a chimeric repressor of *TCP3* (*TCP3SRDX*) is expressed, and this phenotype is partly suppressed by the inactivation of the *CUC1* or *CUC2* gene (Koyama *et al.*, 2007). This suggests that part of the *TCP3SRDX* phenotype is due to increased *CUC* activity, which could be correlated to a lower miR164 expression. Furthermore, when miR164 regulation of *CUC2* is reduced, leaf size is increased as it is in plants with weaker *TCP* activity (Efroni *et al.*, 2008; Schommer *et al.*, 2008; Larue *et al.*, 2009). These observations clearly hint at an exciting connection between the miR319/*TCP* and miR164/*CUC* regulatory modules.

miR319/miRJAW shares a high sequence identity (17 of 21 nt) with another miRNA, miR159. Indeed, when overexpressed, miR319/miRJAW can regulate the miR159 targets *MYB33* and *MYB65* (Palatnik *et al.*, 2007). Conversely, the reduced sequence complementarity between miR159 and the *TCP* genes prevents miR159 from targeting these genes, and this absence of regulation is exemplified by the lack of the ‘jaw’ phenotype in miR159 overexpressors (Achard *et al.*, 2004; Palatnik *et al.*, 2007). However, miR159 has a role in leaf development. Double mutants of the *mir159a* and *mir159b* genes, or plants expressing a miR159-resistant *MYB33* target, have up-curved leaves (Palatnik *et al.*, 2003; Millar and Gubler, 2005; Allen *et al.*, 2007). Although the mechanism by which the miR159/*MYB* regulatory unit determines leaf shape has not been further characterized, gibberellins are possibly involved as both *mir159/MYB* and leaf development are regulated by these hormones (Achard *et al.*, 2004; Hay *et al.*, 2004).

miR396, which belongs to a family of miRNAs identified in both dicots and monocots, also plays a role in leaf growth regulation (Liu *et al.*, 2009). miR396 is encoded by two loci in *Arabidopsis* and targets seven out of the nine *GRF* (*GROWTH REGULATING FACTORS*) genes. The *GRF* genes regulate leaf size via modulation of cell proliferation and cell expansion (Kim *et al.*, 2003; Kim and Kende, 2004; Horiguchi *et al.*, 2005). *miR396* overexpression leads to narrower leaves with fewer cells of larger size, a phenotype resembling multiple *grf* mutants. Although this indicates that the *GRF* genes are targeted *in planta* by miR396, the role of this regulation during leaf growth has still to be clarified.

## An miRNA controls stomatal patterning during leaf differentiation

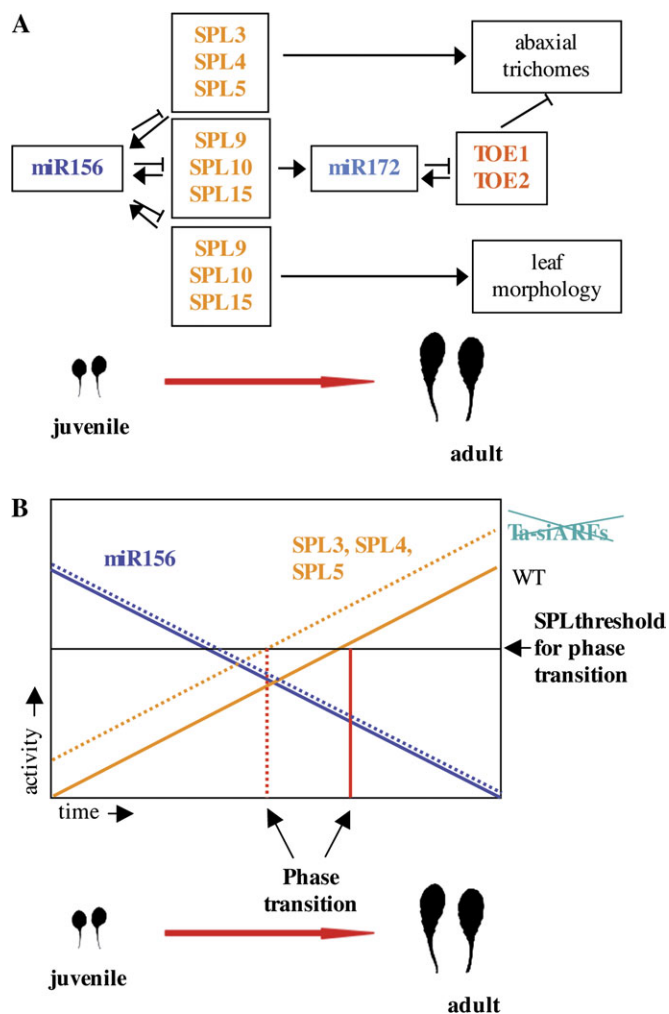
Leaves are the main photosynthetic organs of the plants and they bear specialized cell types, the stomata, to optimize gas exchange with the environment. A new function in stomata development has been described for miR824 and its unique target, the MADS gene *AGL16* (*AGAMOUSLIKE 16*) (Kutter *et al.*, 2007). miR824 is an miRNA specific to the Brassicaceae and is likely to have evolved recently in this lineage through duplication and rearrangement of the *AGL16* sequence (Rajagopalan *et al.*, 2006; Fahlgren *et al.*, 2007; Kutter *et al.*, 2007). Stomata originate through asymmetric divisions of meristemoid cells, which give rise to epidermal cells and a guard mother cell that, in turn, divides symmetrically to produce two guard cells (for a review, see Bergmann and Sack, 2007). In addition to these primary stomatal complexes, there are also secondary and tertiary higher order stomatal complexes, which derive from satellite meristemoids. Stomatal density, and specifically the frequency of these higher order stomatal complexes, is increased when the regulation of *AGL16* by miR824 is impaired, whereas, conversely, lower *AGL16* levels lead to simplified stomatal complexes. Based on these observations, *AGL16* may promote entry into division in

the satellite meristemoid lineage and/or prevent their differentiation into epidermal pavement cells. miR824 and *AGL16* show complementary expression patterns in the stomatal complexes: miR824 accumulates in satellite meristemoids and in guard mother cells but not in mature guard cells that express *AGL16* mRNA. How *AGL16*, which is normally not expressed in the meristemoids, may modify their behaviour is unknown. It has been proposed that *AGL16* may diffuse from immature guard cells to neighbouring cells (Kutter *et al.*, 2007). Alternatively, *AGL16* may be a signal promoting stomata differentiation that is normally excluded from the satellite meristemoids by miR824, and ectopic *AGL16* expression in these cells, in the case of defective miR824 regulation, could trigger their differentiation into stomata.

In addition to their modified stomata complexes, plants expressing an miR824-resistant *AGL16* have more leaves of smaller size and abnormal morphology, including twisting and up-curling of the margin. Together, these data show that restriction of *AGL16* to the stomata lineage by miR824 is important for normal leaf development.

## Phase transition

Plants undergo multiple changes during their life cycle, including two phase transitions: from the juvenile to adult phase during vegetative development, and, afterwards, from the vegetative to the reproductive phase. The transition from the juvenile to the adult phase includes different changes in leaf morphology or leaf heteroblasty. In the case of *Arabidopsis*, phase transition is reflected by an increase in petiole length, lamina length/width ratio, and serration, by more cells but of smaller size, and the appearance of trichomes on the abaxial side (Telfer *et al.*, 1997; Tsukaya *et al.*, 2000; Usami *et al.*, 2009). Two miRNAs, miR156 and miR172, are involved in the phase transition in *Arabidopsis* and maize (Figs 2, 4A) (Aukerman and Sakai, 2003; Chen, 2004; Lauter *et al.*, 2005; Wu and Poethig, 2006; Chuck *et al.*, 2007; Schwarz *et al.*, 2008; Wu *et al.*, 2009). MiR156 is a master regulator in this process, as it is both necessary and sufficient to promote juvenile identity, and integrates several partially overlapping pathways controlling leaf shape and differentiation. First, the *SPL9/SPL10/SPL15* genes control changes in leaf size, shape, and serration, possibly via the regulation of cell size and number (Schwarz *et al.*, 2008; Usami *et al.*, 2009; Wu *et al.*, 2009). Secondly, these genes also control the formation of abaxial trichomes. Interestingly, *SPL9* has been shown to activate directly the expression of *MIR172b*. This miRNA promotes vegetative adult identity via the negative regulation of two *AP2* genes, *TOE1* and *TOE2*. Finally, the *SPL3/SPL4/SPL5* genes also control trichome formation, though independently of *miR172*. Since it was observed that during vegetative maturation there is a decrease of miR156 that correlates with an increasing expression of miR172, the following model has been proposed. During the juvenile phase, high levels of miR156 maintain *SPL* gene activity low, which



**Fig. 4.** Model for the role of miR156, miR172, ta-siARFs and their respective targets in the control of vegetative phase transition. (A) MiR156 regulation of different *SPL* genes coordinates the different changes of leaf characteristics that are associated with the juvenile to adult phase transition. MiR156 regulates the formation of trichomes in the abaxial epidermis via two overlapping pathways, *SPL3/SPL4/SPL5* and *SPL9/SPL10/SPL15*. In the latter case, *SPL9/SPL10* function involves another miRNA, miR172, and its targets *TOE1* and *TOE2*. miR156 regulation of *SPL9/SPL10/SPL15* also controls changes in leaf morphology via an alternative pathway independent of miR172. See text for further details. (B) Model for the contribution of the ta-siARFs to the juvenile to adult phase transition. In mutants of the ta-siARF biosynthesis machinery (dotted lines), *SPL3/SPL4/SPL5* activity levels are increased and reach, before the wild-type, a putative threshold triggering the phase transition. Changes in *SPL3/SPL4/SPL5* are not due to modified miR156 activity in the mutants. The effect of the ta-siARFs on *SPL3/SPL4/SPL5* but not on *SPL9/SPL10/SPL15* explains the earlier formation of abaxial trichomes but not the earlier changes in leaf shape of ta-siRNA production mutants.

prevents miR172 activation and leads to *TOE1* and *TOE2* expression. As miR156 decreases, elevated *SPL* activities promote adult fate, either directly or via the activation of miR172, which in turn represses the *TOE1* and *2* genes. In this view, the miR156/*SPL* regulatory unit provides

a quantitative developmental clock. How the pace of this clock is set, and, in particular, how the decrease in miR156 levels is controlled currently remain unknown. In addition to this interplay between two miRNAs, negative feedback loops between the targets and their regulatory miRNA have also been shown. These negative feedback loops may contribute to stabilize the expression level of the target, but could also be a way to modulate miR156 levels during development. Finally, the decline in miR156 and the rise of the *SPL* factors and miR172, which promote the adult phase, also promote the transition to the reproductive phase (Aukerman and Sakai, 2003; Chen, 2004; Wang *et al.*, 2009), underlying their essential contribution to plant phase transition and coordination.

Ta-siARFs are also involved in vegetative phase transition (Peragine *et al.*, 2004; Fahlgren *et al.*, 2006; Hunter *et al.*, 2006). Mutants of the ta-siRNA biogenesis pathway such as *ago7*, *rdr6*, and *dcl4*, and plants expressing a ta-siRNA-resistant *ARF3* gene show an accelerated transition to the adult phase. In contrast, mutants in the ta-siARF targets *ARF3/ETT* and *ARF4* show a delayed phase transition. Altogether, this indicates that *ARF3/ETT* and *ARF4* activities, determined partly by the ta-siARFs, control, in a quantitative manner, the speed of the juvenile to adult phase transition. How *ARF3/ETT*, *ARF4*, and *TAS3* regulate this transition, however, is not clear. Neither the ta-siARFs nor the *ARF3* and *ARF4* transcript levels are modified during vegetative development in wild-type plants, suggesting that they are not part of the developmental clock regulating the transition. A possible mechanism links the ta-siARF pathway with some *SPL* genes (Fig. 4B). In ta-siRNA production mutants such *ago7* and *rdr6*, *SPL3/SPL4* mRNA levels progressively increase during development like in the wild type, but their overall level is higher (Peragine *et al.*, 2004; Wu and Poethig, 2006). Assuming that the transition to the adult phase is achieved when *SPL3/4* activity reaches a threshold level, this would explain the accelerated transition of *ago7* and *rdr6* mutants to the adult phase. Interestingly, higher *SPL3/SPL4* levels in *ago7* and *rdr6* do not rely on a lower miR156 level or activity (Peragine *et al.*, 2004; Wu and Poethig, 2006), suggesting that other mechanisms, possibly an increased transcription of *SPL* genes, may operate. This simple model, nonetheless, does not fully explain the *ago7* and *rdr6* phenotypes. These mutants show not only earlier abaxial trichomes, which is explained by the higher *SPL3/SPL4* levels, but also earlier changes in leaf shape that do not rely on the *SPL3/SPL4* genes but on higher *SPL9/SPL10* activities (Fig. 4A). Higher *SPL9/SPL10* expression levels, however, are not observed in *ago7* and *rdr6* mutants (Wu and Poethig, 2006). Therefore, the ta-siARFs may also control phase transition via a mechanism that does not involve changes in *SPL* expression. One option could be that ta-siARFs modulate the temporal signal provided by the developmental clock involving miR156/*SPL*, possibly with auxin at the nexus of this interaction, as the *SPL* factors may modify auxin signalling and the ARF proteins are involved in auxin response.

## Leaf senescence

The last stage in leaf life is senescence, which includes very different processes such as the exportation of nutrients to other tissues, chlorophyll degradation, and finally cell death. Two miRNAs regulate different mechanisms entailing leaf ageing and senescence. These two miRNAs, miR319 and miR164, also regulate previous stages of leaf development such as initiation, growth, and differentiation. In this way, they could provide a way to coordinate the different steps of a leaf's life (Figs 2, 5).

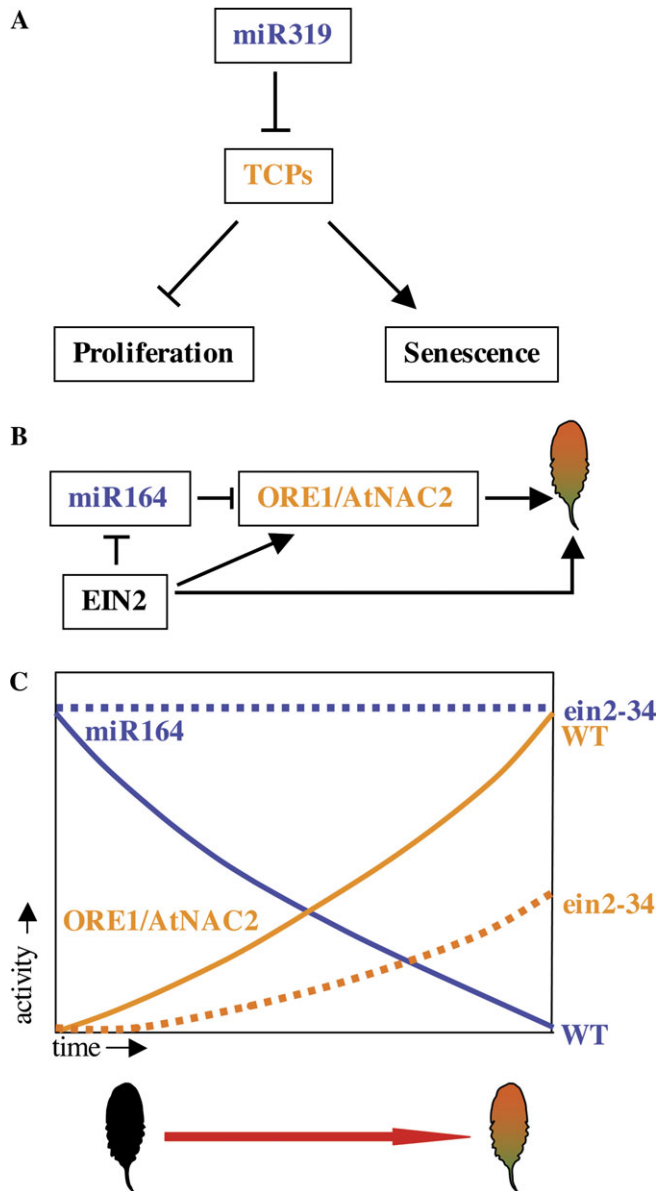
Several hormones regulate leaf senescence, including salicylic acid, ethylene, cytokinins, and JA (jasmonic acid) (van der Graaff *et al.*, 2006). Recently, miR319/miR164 has been implicated in the control of leaf senescence (Schommer *et al.*, 2008). TCPs regulated by miR319 control JA biosynthesis by modulating JA biosynthetic genes, in particular *LOX2*, a chloroplast-localized lipoxygenase that catalyses the conversion of  $\alpha$ -linolenic acid into (13S)-hydroperoxyoctadecatrienoic acid, the first step in the biosynthesis of the oxylipin JA. Since miR319/TCP regulation of *LOX2* is not related to stress stimuli or wounding, it seems that the TCPs regulated by miR319/miR164 only control developmental aspects of *LOX2* expression. Although JA apparently is not essential for senescence, Schommer *et al.* (2008) suggest that in parallel to JA biosynthesis regulation, miR319/TCPs regulate other genes such as *WRKY53*, an important positive regulator of senescence. This would involve the miR319/TCPs axis in a second parallel pathway, suggesting a more general role for TCPs in leaf ageing and senescence. In this way, miR319 negatively regulates leaf growth and positively regulates leaf senescence by modulating the activity of TCP transcription factors (Fig. 5A).

Finally, a new function has also been described for miR164 in the last stage of leaf life (JH Kim *et al.*, 2009). In addition to the *CUC1* and *CUC2* genes, miR164 regulates the *Arabidopsis* NAC transcription factor *ORE1* (ORE-SARA1, oresara means 'long living' in Korean), a positive regulator of ageing-induced cell death and senescence in *Arabidopsis* leaves (Fig. 5B,C). Whereas miR164 progressively decreases during leaf ageing, *ORE1* expression increases. Down-regulation of miR164 is mediated by *EIN2* (*ETHYLENE INSENSITIVE2*), which is a central component of ethylene signalling. Independently of miR164, *EIN2* also induces an age-dependent up-regulation of *ORE1*. Finally, *EIN2* also regulates ageing-induced cell death by a pathway independent of both miR164 and *ORE1*. These feed-forward regulations ensure coupled senescence and cell death when leaves age. In such a network, miR164 may prevent premature overexpression of *ORE1* to fine-tune the timing of senescence and cell death.

## Conclusion

Since the identification of the first plant miRNAs in 2002, considerable progress has been made in the understanding





**Fig. 5.** miR164 controls senescence by regulating *ORE1/AtNAC2*. (A) miR319 targeting of *TCP* genes coordinates different phases of leaf development by negatively regulating proliferation and positively regulating senescence. (B) *ORE1/AtNAC2*, involved in cell death and senescence, is negatively regulated by miR164 and positively regulated by *EIN2*, which also down-regulates miR164. *EIN2* also induces senescence by another pathway independent of miR164/*ORE1*. (C) *ORE1/AtNAC2* and miR164 activities during leaf development in the wild type and *the ein2-34* mutant. In the wild type, *EIN2* inhibition of miR164 leads to a decrease of its activity during leaf ageing, which allows the level of *ORE1/AtNAC2* to increase. In the *ein2-34* mutant, miR164 levels are stable during leaf ageing and the levels of *ORE1/AtNAC2* are lower, which delays leaf senescence.

of their role during development, such as those illustrated here for the leaf. It is clear now that miRNAs and ta-siRNAs are not only quantitative or qualitative regulators of gene expression, but also have an important role in complex gene regulatory networks.

Because of their mode of action, miRNAs and ta-siRNAs can target simultaneously, and hence coordinate, the expression of several genes, typically of the same family. This allows the coordination of genes with redundant roles such as the *TCP* or *CUC* genes. However, sometimes targets of the same miRNA regulate different processes, such as in the case of the targets of miR164 that regulate leaf morphogenesis or leaf senescence. Therefore, miRNA and ta-siRNA appear to have a prominent role in the temporal coordination of successive events occurring at the plant or organ level, such as phase transition or the switch from morphogenesis to senescence.

MiRNAs and ta-siRNAs (and sometimes even the same molecule) can have both a very local role (such as for the ta-siARFs in the control of leaf polarity, or miR164 in the control of serration) and regulate processes at the whole organ or even plant scale (such as ta-siARFs during phase transition or miR164 during leaf senescence). How these different levels of action are coordinated is unknown. One possibility would be specialization of miRNA gene function, and important issues for the future will be to determine the role of the multiple genes that often code for similar miRNAs, the signification of the minor sequence divergence within a miRNA family, and why miRNAs often target only some members of a gene family.

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