Plant microRNAs and development

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ABSTRACT **MicroRNAs (miRNAs) act as negative regulators of gene expression in eukaryotes, a discovery that has opened an expanding field of biological research. Plant miRNAs are known to repress gene expression posttranscriptionally, mainly by guiding cleavage but also by attenuating the translation of target transcripts. In addition, it has been shown that plant miRNAs can also act at the transcriptional level by directing the methylation of target chromosomal loci. Genetic and biochemical approaches are quickly broadening our knowledge of the biogenesis and function of plant miRNAs. Computational approaches have uncovered an unexpectedly large number of miRNAs and their targets in plants. The targets of plant miRNAs often belong to families of transcription factors involved in the control of developmental processes. We review the status of research in this dynamic field, summarizing recent advances in our understanding of the biogenesis and mechanism of action of plant miRNAs, as well as in the developmental processes they regulate.**

KEY WORDS: *microRNA, Arabidopsis, plant development*

History of miRNA research

Almost twenty years after the isolation of the *lin-4* heterochronic mutant of *Caenorhabditis elegans*, the corresponding gene was cloned (Lee *et al.*, 1993). Genetic and molecular analyses identified *lin-4* as a negative regulator of *lin-14* (Ambros, 1989; Arasu *et al.*, 1991). Strikingly, the *lin-4* gene lacked an open reading frame and was located in an intron of an apparently unrelated gene. However, it was highly conserved across nematodes and encoded two small untranslated transcripts of 22 and 61 nucleotides (*lin-4S* and *lin-4L*). The sequence of the *lin-4S* transcript was internal to that of *lin-4L* and complementary to seven repeats of the 3' UTR of *lin-14*. Based on additional genetic and biochemical evidence, a model was proposed to explain the repression exerted by *lin-4* on *lin-14*. According to this model, the *lin-4S* transcripts were able to block the translation of the *lin-14* mRNA by annealing to complementary sequences in its 3' UTR (Lee *et al.*, 1993; Wightman *et al.*, 1993; Olsen and Ambros, 1999). Support for this model came from the ability of the 3' UTR of *lin-14* to confer the temporal expression pattern of *lin-14* to unrelated RNAs (Wightman *et al.*, 1993). *lin-4* was considered a rarity until February 2000, when the *let-7* gene was also identified in *Caenorhabditis elegans* (Reinhart *et al.*, 2000). Similar to *lin-4*, *let-7* was found to encode another small RNA that negatively regulated genes involved in the developmental timing of the nematode (Reinhart *et al.*, 2000). The small RNA products of *lin-4* and *let-7* acted as translational repressors by binding to the 3' UTRs of their target mRNAs. Conservation across bilaterians was found for *let-7*, suggesting a more extensive role for miRNAs in gene regulation (Pasquinelli *et al.*, 2000).

Studies on the biosynthesis of flavonols and anthocyanins led to the discovery of co-suppression in plants (Napoli *et al.*, 1990; Van der Krol *et al.*, 1990). This phenomenon was discovered as an unexpected result of experiments aimed at overexpressing the chalcone synthase and dihydroflavonol 4-reductase genes in petunia. Rather than higher expression levels of the genes under study, reduced RNA levels for both the transgene and the endogenous gene were obtained. Among other hypotheses, DNA methylation and the interference of transcription by RNA strands were proposed as possible mechanisms for the observed trans-acting gene silencing (Napoli *et al.*, 1990; Van der Krol *et al.*, 1990). After the finding, in 1998, that the activity of any given gene could be posttranscriptionally interfered in a highly specific and efficient manner by injecting double-stranded RNAs in *Caenorhabditis elegans* (Fire *et al.*, 1998; Montgomery *et al.*, 1998), RNA interference (RNAi) became one of the preferred techniques to knock down gene expression in many organisms (reviewed by Hammond *et al.*, 2001).

The link between these apparently unrelated silencing phenomena was apparent after finding that they are mediated by similar short non-coding RNAs, known as microRNAs (miRNAs) and short interfering RNAs (siRNAs), and processed by similar

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Abbreviations used in this paper: miRNA, microRNA.

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enzymes, which are highly conserved from yeast to metazoans and plants (Bernstein *et al.*, 2001; Grishok *et al.*, 2001; Ketting *et al.*, 2001). Distinction of these small RNA species is mainly based on their expression and biogenesis (Ambros *et al.*, 2003a). In this review, we will focus on the most recent advances on the biogenesis and mode of action of plant miRNAs, as well as on their role in plant development.

The biogenesis and export of microRNAs

miRNAs are small RNAs of approximately 22 nucleotides in length, which derive from non-protein coding transcripts and function as developmentally regulated repressors of gene expression in eukaryotes. Plant and animal miRNAs are diverse in sequence, abundance, expression pattern and genomic location (Lee and Ambros, 2001; Reinhart *et al.*, 2002; Bartel, 2004; Griffiths-Jones, 2004; Griffiths-Jones *et al.*, 2005). Whereas most plant miRNAs derive from their own loci, some reside in introns of other protein-coding genes and in the same orientation, suggesting that they are transcribed in coordination with their host genes (Bartel, 2004; He and Hannon, 2004). Because the annotation of eukaryotic genomes has mainly focused on the thousands of protein-coding genes, miRNA genes are located in regions previously annotated as intergenic, often clustered as polycistronic units (Ambros, 2004; Bartel, 2004).

Studies in plant and animals have shown that miRNA genes are transcribed by RNA polymerase II from their own promoters (Cai *et al.*, 2004; Kurihara and Watanabe, 2004; Lee *et al.*, 2004; Parizotto *et al.*, 2004). As is characteristic of class II genes, the primary transcripts of miRNA genes (also known as pri-miRNAs; Lee *et al.*, 2002) may contain introns and are polyadenylated and 5' capped (Aukerman and Sakai, 2003; Cai *et al.*, 2004; Kurihara and Watanabe, 2004; Lee *et al.*, 2004; Parizotto *et al.*, 2004). These long pri-miRNAs are predicted to adopt imperfect stem-loop (hairpin) secondary structures that contain the miRNA in one arm of the stem (Lee *et al.*, 2002; Reinhart *et al.*, 2002). The folding of pri-miRNAs is essential for their processing by class III ribonucleases (RNase III), which cleave them into smaller miRNA precursors (also termed pre-miRNAs; Lee *et al.*, 2003; Zeng *et al.*, 2005). In animals, this task is carried out in the nucleus by the microProcessor protein complex, which includes the RNase III Drosha and the dsRNA-binding protein Pasha/DGCR8 (Denli *et al.*, 2004; Gregory *et al.*, 2004; Han *et al.*, 2004a; Landthaler *et al.*, 2004). In animals, the cleavage of pri-miRNAs into pre-miRNAs and the cleavage of pre-miRNAs into miRNA-miRNA* duplexes are mediated by the RNase III activities of Drosha in the nucleus (Lee *et al.*, 2003) and Dicer in the cytoplasm (Billy *et al.*, 2001; Provost *et al.*, 2002), respectively, making the nucleocytoplasmic export of the pre-miRNAs an essential step in miRNA biogenesis (Lee *et al.*, 2002; Lund *et al.*, 2004). The miRNA and miRNA* strands of the miRNA-miRNA* duplexes originate from complementary arms of the stem, which remain together after cleavage, leaving 2-nucleotide 3' overhangs with 5' monophosphate and 3' hydroxyl ends, as is characteristic of Dicer activity (Elbashir *et al.*, 2001).

Our current knowledge on the biogenesis of plant miRNAs comes from the characterization of *Arabidopsis thaliana* mutants showing defective miRNA biogenesis. In this species, the *DICER-LIKE1* (*DCL1*) gene was initially identified based on the developmental defects shown by loss-of-function mutants isolated in a variety of screens. These included embryo defects caused by the *suspensor1* alleles of *DCL1* (*sus1*; Errampalli *et al.*, 1991; Castle *et al.*, 1993; Schwartz *et al.*, 1994; Franzmann *et al.*, 1995; McElver *et al.*, 2001), aberrant female reproductive development, extra vegetative leaves and inflorescence axes and late flowering caused by the *short integuments1* alleles (*sin1*; Robinson-Beers *et al.*, 1992; Lang *et al.*, 1994; Ray *et al.*, 1996) and aberrant floral patterning, fewer axillary meristems and abnormal proliferation of shoot meristem cells caused by the *carpel factory* allele (*caf*; Jacobsen *et al.*, 1999; Figure 1C). The involvement of *DCL1* in miRNA biogenesis was proposed after reduced miRNA accumulation was observed in the *caf* mutant (Park *et al.*, 2002; Reinhart *et al.*, 2002). Loss-of-function mutations of two additional *Arabidopsis* genes, *HYPONASTIC LEAVES1* (*HYL1*) and *HUA ENHANCER1* (*HEN1*), condition pleiotropic phenotypes similar to those of *dcl1* alleles, including small lateral organs, hyponastic (curled up) vegetative leaves with a gradual lamina-to-petiole transition and abaxial trichomes, dense inflorescences, hook-shaped cauline leaves, short stature, delayed flowering and low fertility (Figure 1D, E; Lu and Fedoroff, 2000; Chen *et al.*, 2002; Han *et al.*, 2004b; Vazquez *et al.*, 2004; Jover-Gil *et al.*, submitted). Similar to *dcl1*, *hyl1* and *hen1* mutations impair the production of miRNAs and lead to a reduced accumulation of miRNAs, suggesting that these genes are also required for miRNA biogenesis (Park *et al.*, 2002; Boutet *et al.*, 2003; Han *et al.*, 2004b; Vazquez *et al.*, 2004).

In *Arabidopsis thaliana*, the cleavage of pri-miR163 into pre-miR163 is carried out in two steps by the RNase III activity of DCL1, as inferred from the accumulation of pri-miR163 and the absence of cleavage products resulting from a mutation that damages the RNA helicase domain of DCL1 (Kurihara and Watanabe, 2004). It has recently been hypothesized that plants have an analogous complex to the microProcessor of *Drosophila*, which would include DCL1 and the dsRNA-binding protein (HYL1), since HYL1 forms part of a complex whose molecular weight coincides with those of HYL1 and DCL1 together (Han *et al.*, 2004b; Tomari and Zamore, 2005). Unlike animals, the cleavage of plant pre-miRNAs into shorter miRNA-miRNA* duplexes is also carried out by DCL1, making it conceivable that all the steps happen in the nucleus (Papp *et al.*, 2003; Kurihara and Watanabe, 2004; Park *et al.*, 2005). In agreement with the nuclear localization of plant miRNA biogenesis, DCL1, HYL1 and HEN1 contain nuclear localization signals (Jacobsen *et al.*, 1999; Lu and Fedoroff, 2000; Chen *et al.*, 2002) and mature miRNAs have been found in the nucleus and, more abundantly, in the cytoplasm, where they may function (Park *et al.*, 2005). In mammalian cells, the export of pre-miRNAs from the nucleus to the cytoplasm is accomplished by Exportin-5 in cooperation with Ran-GTP (Yi *et al.*, 2003; Bohnsack *et al.*, 2004; Lund *et al.*, 2004). HASTY (HST; Telfer and Poethig, 1998), the plant ortholog of Exportin-5, interacts with AtRAN1 (Bollman *et al.*, 2003) and Park and colleagues (2005) have shown that a missense *hst-3* mutation, known to preclude the interaction with Ran-GTP, impairs the accumulation of miRNAs, pointing to the involvement of HST in miRNA transport or biogenesis. Consistent with this role, they also found that the transcript levels of several miRNA targets were higher in a putatively null *hst* mutant (Park *et al.*, 2005). Mutations in this gene also cause developmental defects, such as the curling-up of the leaves, early

flowering and an accelerated vegetative phase change (Figure 1F; Telfer and Poethig, 1998).

Insight into the role of *HEN1* in miRNA biogenesis has been gained recently with the finding that HEN1 is a methyltransferase that methylates miRNA-miRNA* duplexes, but not single stranded miRNAs, miRNA*s, siRNA duplexes, or DNA duplexes (Yu *et al.*, 2005). Whereas the biological significance of this methylation is still unclear, Yu and colleagues have proposed two hypotheses to account for the observed decrease in the amount of miRNAs in *hen1* mutants. According to one of them, the methyl group may act as a protective cap to prevent the degradation of miRNAs by exonucleases. Alternatively, the methyl group might serve as a recognition signal to direct the miRNAs to subsequent steps of the miRNA pathway, which may also increase the stability of miRNAs (Yu *et al.*, 2005).

Posttranscriptional regulation by miRNAs

Plant miRNAs negatively regulate endogenous target genes whose transcripts are recognized and cleaved based on their extensive or complete complementarity (Rhoades *et al.*, 2002; Bartel and Bartel, 2003). As expected, mutations that impair the biogenesis of miRNAs, such as *hyl1*, *hen1* and *hst*, lead to the upregulation of genes that are targeted by miRNAs (Boutet *et al.*, 2003; Han *et al.*, 2004b; Vazquez *et al.*, 2004; Park *et al.*, 2005). In plants, each target mRNA usually contains a single miRNA complementary site located in the coding region (Rhoades *et al.*, 2002; Llave *et al.*, 2002a; Bartel, 2004). In contrast, animal miRNAs are often partially complementary to multiple binding sites in the 3' UTR of the target mRNAs (Lee *et al.*, 1993; Wightman *et al.*, 1993; Olsen and Ambros, 1999; Seggerson *et al.*, 2002; Bartel, 2004). Different from most plant miRNAs and similar to animals, miRNA172 is the only plant miRNA known to act by repressing the translation of its targets (Aukerman and Sakai, 2003; Chen, 2004).

In a variety of eukaryotes, the endonuclease activity of the RNA-Induced Silencing Complex (RISC) cleaves target RNA molecules guided by complementary miRNAs or siRNAs (Hammond *et al.*, 2000; Elbashir *et al.*, 2001; Zamore, 2002). The RISC complex characteristically contains a member of the ARGONAUTE (AGO) or PPD protein family, which provides the endonuclease (sometimes referred to as 'slicer') activity of the complex (Liu *et al.*, 2004; Meister *et al.*, 2004; Rand *et al.*, 2004). AGO proteins contain highly conserved PAZ and PIWI domains (Cerutti *et al.*, 2000; Carmell *et al.*, 2002). The PAZ domain binds

Fig. 1. Perturbation of the miRNA pathway causes aberrant rosette and leaf morphology. (A,B) *Rosettes of the wild-type Landsberg* erecta *(L*er*; A) and Enkheim-2 (En-2; B) accessions.* **(C-G)** *Rosette phenotypes caused by loss-of function mutations in the* DCL1 *(C),* HEN1 *(D),* HYL1 *(E),* HST *(F) and* AGO1 *(G) genes, all of which encode proteins of the miRNA machinery.* **(H)** *Morphological effects of a semidominant, point mutation that damages the miR166 binding site of* ICU4 *(also known as* CNA *and* ATHB-15 *). The* ago1*,* dcl1*,* hen1 *and* hyl1 *mutations are in a* Ler *genetic background, whereas* hst *and* icu4 *are in a En-2 genetic background.* **(I-N)** *Synergistic phenotypes of some double mutants involving* ago1*,* dcl1*,* hen1*,* hyl1*,* hst *and* icu4*. Pictures were taken in our laboratory from plants grown at 20°C on agar plates under continuous light, 21 (A, B, D-J, L, N), 25 (M), 26 (C) and 37 (K) days after sowing. Scale bars indicate 0.2 mm (L) and 1 mm (A-K, M, N).*
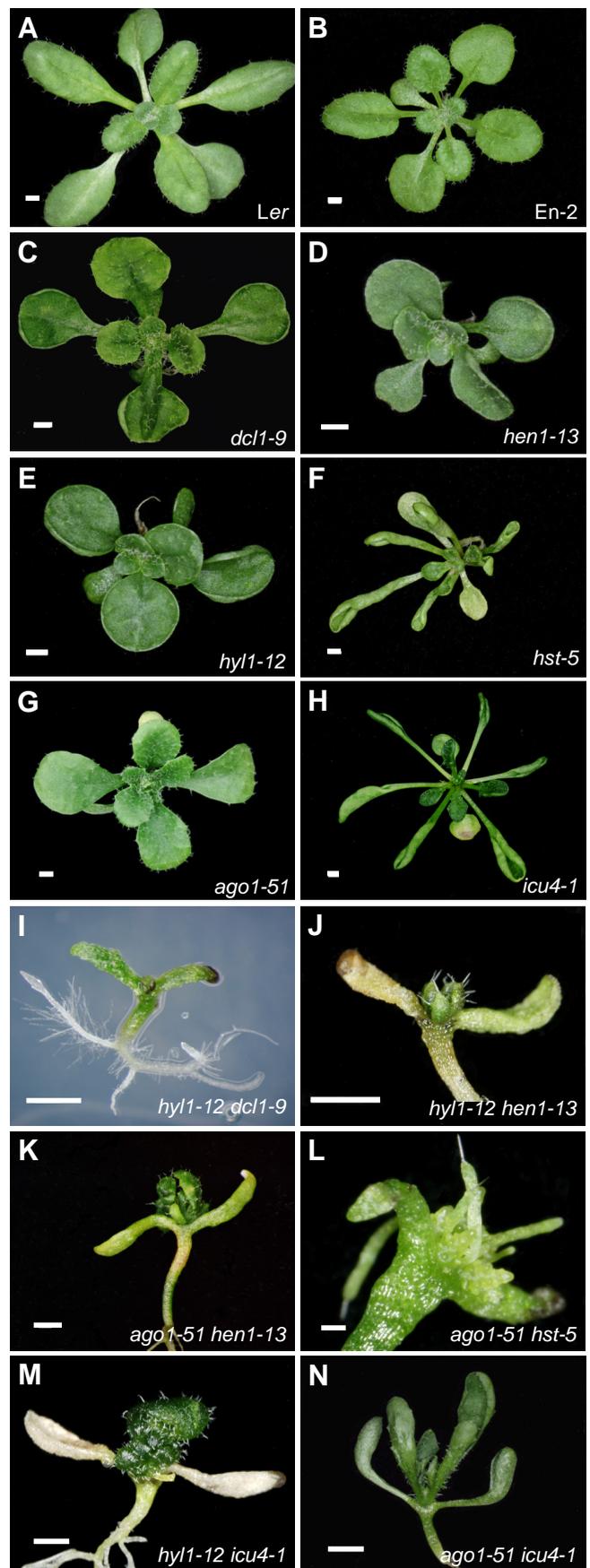
single-stranded RNAs and, with lower affinity, dsRNAs with 3' overhangs (Lingel *et al.*, 2003; Yan *et al.*, 2003). The PIWI domain mediates the interaction with Dicer proteins and provides endonuclease activity (Liu *et al.*, 2004; Parker *et al.*, 2004; Song *et al.*, 2004; Tahbaz *et al.*, 2004). The cleavage of targets occurs at the 10th or 11th nucleotide from the 5' end of the miRNA, regardless of the miRNA length, and requires the base pairing of the 5' end of the miRNA with the mRNA (Palatnik *et al.*, 2003; Xie *et al.*, 2003; Floyd and Bowman, 2004; Jones-Rhoades and Bartel, 2004; Mallory *et al.*, 2004a). The resulting cleavage products have 3' hydroxyl and 5' phosphate groups, similar to the products of other enzymes with 'slicer' activity, such as RNase H (Martinez and Tuschl, 2004; Schwarz *et al.*, 2004; Song *et al.*, 2004).

ARGONAUTE1 (*AGO1*; Bohmert *et al.*, 1998) encodes one of the ten AGO proteins present in the genome of *Arabidopsis thaliana* (Morel *et al.*, 2002). Several facts suggest that AGO1 forms part of the plant RISC in the miRNA pathway. First, miRNA target genes are upregulated in loss-of-function *ago1* mutants, even though miRNA levels are not reduced suggesting that *AGO1* acts downstream *DCL1*, *HYL1* and *HEN1* (Kidner and Martienssen, 2004; Vaucheret *et al.*, 2004). Second, several amino acid residues that are conserved in AGO1 have been shown to be essential for the endonuclease activity of other AGO proteins (Liu *et al.*, 2004; Song *et al.*, 2004). Third, the misregulation of endogenous genes due to a faulty miRNA pathway is expected to cause developmental phenotypes, such as those of *ago1* mutants (Figure 1G). Null *ago1* mutants are sterile and show loss of lateral organ polarity and defective axillary meristem development (Bohmert *et al.*, 1998). In addition, posttranscriptional gene silencing was found to be altered in *ago1* mutants, which suggested that their developmental phenotypes derive from altered gene regulation (Fagard *et al.*, 2000). The pleiotropic phenotype conditioned by weaker, hypomorphic *ago1* alleles includes the adaxialization of lateral organs, the presence of abaxial trichomes on juvenile leaves, a loss of lamina-petiole distinction, reduced flower size, late flowering, abnormal inflorescence morphology and small stature (Figure 1G; Morel *et al.*, 2002; Kidner and Martienssen, 2004; Jover-Gil *et al.*, submitted). Some of these phenotypes resemble those of hypomorphic *dcl1* and null *hen1* and *hyl1* mutants (Figure 1C-E, G; Hunter and Poethig, 2003; Vaucheret *et al.*, 2004; Vazquez *et al.*, 2004; Jover-Gil *et al.*, submitted) and are enhanced in double mutants involving mutations affecting the miRNA pathway (Figure 1I-L; Jover-Gil *et al.*, submitted), as is expected if AGO1 is a component of the miRNA pathway.

An interesting discovery was that *DCL1* and *AGO1* are regulated by miRNAs (Table 1; Xie *et al.*, 2003; Vaucheret *et al.*, 2004; Vazquez *et al.*, 2004), which indicates that the miRNA pathway is subjected to a negative feedback by miRNAs. As expected for a target of miRNA cleavage, *AGO1* was upregulated in *dcl1* and *hen1* mutants (Vaucheret *et al.*, 2004; Vazquez *et al.*, 2004). In addition, plants carrying a miR168-resistant version of *AGO1* (miR168 targets *AGO1*) also showed an upregulation of *AGO1*, which correlated with the severity of the developmental defects displayed (Vaucheret *et al.*, 2004). These phenotypes were similar to those displayed by *hyl1*, *dcl1* and *hen1* mutants. The upregulation of *AGO1* was also observed in the null *ago1-3* mutant, consistent with AGO1 being both a component of the plant RISC and a target of miRNA regulation (Vaucheret *et al.*, 2004).

After the target mRNAs are cleaved by the RISC, the resulting 3' and the 5' cleavage products follow different fates. In *Arabidopsis thaliana*, the 3' fragments are degraded through the activity of the cytoplasmic 5'-to-3' EXORIBONUCLEASE4 (XRN4), as inferred from the increased stability of these fragments in loss-of-function *xrn4* mutants (Souret *et al.*, 2004). The ortholog of *XRN4* in *Drosophila*, *XRN1*, is involved in the degradation of the 3' fragments after the cleavage by AGO2 in the RISC, and the degradation of the 5' fragment is accomplished by the cytoplasmic exosome, a 3'-to-5' riboexonuclease complex (Orban and Izaurralde, 2005).

TABLE 1

miRNAs KNOWN TO PLAY A ROLE IN DEVELOPMENT

Organism	microRNA	Approach	Target genes	Function of the target genes	References
C. elegans	lin-4	M	*lin-14, lin-28*	Larval development	1-5
C. elegans	let-7	M	*lin-41, lin-57* (*hbl-1*), *daf-12, pha-4*	Larval and adult development, dauer formation and aging	6-10
C. elegans	lsy-6	M	*cog-1*	Neuron bilateral asymmetric gene expression	11
C. elegans	mir-273	P	*die-1*	Neuron bilateral asymmetric gene expression; regulation of *lsy-6*	12
D. melanogaster	bantam	M	*hid*	Cell proliferation and apoptosis	13
D. melanogaster	miR-14	M	Unknown	Apoptosis and fat metabolism	14
M. musculus	miR-181	CS	Unknown	Hematopoietic lineage differentiation	15
M. musculus	miR-375	CS	*Mtpn*	Insulin secretion	16
A. thaliana	miR156	CD	*SPL2, SPL10*	Floral meristem identity and flowering time	17-20
A. thaliana	miR159	CD	*MYB33, MYB65*	Plant growth, anther development and flowering time	17, 18, 20-24
A. thaliana	miR-JAW	M	*TCP2, TCP3, TCP4, TCP10, TCP24*	Cell division, leaf development and embryo patterning	22
A. thaliana	miR162	CD	*DCL1*	Biogenesis of miRNAs and control of meristem function in flower development	17, 25
A. thaliana	miR164, including eep1 (miR164c)	CD, P, S	*CUC1, CUC2, NAC1, At5g07680, At5g61430*	Control of boundary size in meristems and formation and separation of embryonic, vegetative and floral organs, control of petal number	17-20, 26, 27
A. thaliana	miR165 and miR166, including men1 (miR166a)	CD, M	*PHB, PHV, REV, ATHB8, ICU4* (*ATHB-15, CNA*)	Embryo patterning, postembryonic meristem initiation, axial meristem initiation, vascular development, leaf polarity and meristem size regulation	17, 18, 28-31
A. thaliana	miR168	CD, P	*AGO1*	miRNA pathway and stem cell function and organ formation	17, 18, 20, 32
A. thaliana	miR171	CD, P	*SCL6, SCL6-II, SCL6-III*	Radial patterning in roots	17, 18, 20, 33
A. thaliana	miR172, including eat (miR172a-2)	CD, M	*AP2, TOE1, TOE2, TOE3*	Specification of flower organ identity and flowering time	19, 21, 34, 35

[1]Brenner, 1974; [2]Horvitz and Sulston, 1980; [3]Lee *et al.*, 1993; [4]Wightman *et al.*, 1993; [5]Moss *et al.*, 1997; [6]Reinhart *et al.*, 2000; [7]Slack *et al.*, 2000; [8]Abrahante *et al.*, 2003; [9]Lin *et al.*, 2003; [10]Groβhans *et al.*, 2005; [11]Johnston and Hobert, 2003; [12]Chang *et al.*, 2004; [13]Brennecke *et al.*, 2003; [14]Xu *et al.*, 2003; [15]Chen *et al.*, 2004; [16]Poy *et al.*, 2004; [17]Reinhart *et al.*, 2002; [18]Rhoades, 2002; [19]Kasschau *et al.*, 2003; [20]Vazquez *et al.*, 2004; [21]Park *et al.*, 2002; [22]Palatnik *et al.*, 2003; [23]Achard *et al.*, 2004; [24]Millar and Gubler, 2005; [25]Xie *et al.*, 2003; [26]Mallory *et al.*, 2004b; [27]Baker *et al.*, 2005; [28]Emery *et al.*, 2003; [29]Tang *et al.*, 2003; [30]Mallory *et al.*, 2004a; [31]Kim *et al.*, 2005; [32]Vaucheret *et al.*, 2004; [33]Llave *et al.*, 2002a; [34]Aukerman and Sakai, 2003; [35]Chen, 2004. Abbreviations for experimental approaches are as follows: M: mutant screen; P: computational prediction; CS: cloning of small RNAs; CD: cloning of Dicer cleavage products; S: spontaneous mutation.

In plants, a higher instability has been reported for the 5' cleavage products (Llave *et al.*, 2002a) although the degradation mechanism is still unclear. Shen and Goodman (2004) managed to clone the 5' cleavage products of several miRNA targets from *Arabidopsis thaliana* and from mouse, which led them to the discovery that an oligouridine tail is selectively added to their 3' ends. This post-cleavage uridylation might be responsible for the observed enhancement in the decay of the 5' fragments (Shen and Goodman, 2004).

Transcriptional regulation by miRNAs

Using methylation-sensitive restriction enzymes and bisulfite sequencing, Bao *et al.* (2004) found that the *PHABULOSA (PHB)* and *PHAVOLUTA* (*PHV*) genes of *Arabidopsis thaliana*, two well-characterized targets of miR166, are methylated in most wild-type cells. However, *PHB* and *PHV* are not methylated in cells carrying *phb* and *phv* semidominant mutations that damage the miR166 binding site. To investigate the relationship between gene methylation and miRNAs, the authors generated *phb*/*PHB* and *phv*/*PHV* heterozygous plants by combining wild-type and mutant alleles from different ecotypes, which are distinguishable on the basis of sequence polymorphisms. Remarkably, the wild-type alleles were differentially methylated in these heterozygotes, pointing to a novel mode of action, in which miRNAs must interact with their binding sites to drive the methylation of chromosomal loci in *cis*. Because the miRNA binding sites of *PHV* and *PHB* span two exons (Figure 2A), miRNA166 can only interact with spliced *PHV* and *PHB* transcripts, suggesting that the epigenetic modification of these genes happens or is induced before the nascent transcripts are released from the template chromosome (Bao *et al.*, 2004).

This apparently direct interaction between the miRNA and the template chromosome is reminiscent of the silencing of chromatin mediated by the RITS (RNA-induced initiator of transcriptional gene silencing) complex, as noticed by Bao and colleagues (2004). In *Schizosaccharomyces pombe*, the RITS complex contains, among other partners, the AGO protein Ago1 and requires siRNAs produced by Dicer to associate with target loci (Verdel *et al.*, 2004). Recent evidence indicates that the RITS acts in *cis* to allow the maintenance of transcriptional and posttranscriptional silencing and that it might be involved in the processing of nascent transcripts into siRNAs (Noma *et al.*, 2004). Given these mechanistic similarities, it will be interesting to investigate if the methylation and silencing of *PHB* and *PHV* guided by miRNAs depend on protein complexes similar or equivalent to the RITS complex.

Discovering miRNA genes and their targets

Forward genetic approaches based on the isolation of mutants have not been very successful for identifying miRNA genes in plants and animals (Table 1). So far, the only loss-of-function allele of a plant miRNA gene identified is a transposon insertion in the *EARLY EXTRA PETALS1* gene (*EEP1*, also known as *MIR164c*), recently reported by the Meyerowitz lab (Baker *et al.*, 2005). The failure to identify more loss-of-function alleles of miRNA genes might be explained by the difficulty in disrupting their function, since miRNAs are not suitable targets for mutagens because they are small and because they are not affected by frameshift, missense or nonsense mutations (Eddy, 1999). Functional redundancy might

be another cause, since plant genomes usually contain several genes for each miRNA (Llave *et al.*, 2002b; Park *et al.*, 2002; Reinhart *et al.*, 2002; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Wang *et al.*, 2004a; 2004b). In accordance with this, activation tagging approaches have been more efficient in *Arabidopsis thaliana* and have rendered gain-of-function alleles of *JAW* (Palatnik *et al.*, 2003), which was the first plant miRNA gene identified in a mutant screen and, recently, of *EARLY ACTIVATION TAGGED* (*EAT,* also known as *MIR172a-2*; Aukerman and Sakai, 2003) and *MERISTEM ENLARGEMENT1* (*MEN1*, also known as *MIR166a* ; Kim *et al.*, 2005).

The preferential cloning of small RNAs has yielded libraries that include housekeeping RNAs, miRNAs, siRNAs and other small RNAs of unknown function in plants and animals (Elbashir *et al.*, 2001; Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001; Llave *et al.*, 2002b; Park *et al.*, 2002; Reinhart *et al.*, 2002; Ambros *et al.*, 2003b; Aravin *et al.*, 2003; Wang *et al.*, 2004b). New cloning methods that exploit the small size and the biochemical properties (i.e. the 5' phosphate and the 3' hydroxyl groups) of Dicer-dependent cleavage products, first used in animal systems, have been instrumental in the identification of miRNAs (Elbashir *et al.*, 2001; Lau *et al.*, 2001). In *Arabidopsis thaliana*, the selective cloning of short RNAs after ligation of adaptors to the 5' phosphate and the 3' hydroxyl groups has been an effective way of identifying new miRNAs (Llave *et al.*, 2002b; Park *et al.*, 2002; Reinhart *et al.*, 2002).

Some authors, however, have pointed to the limited scope of direct cloning, highlighting the advantages of bioinformatic predictions as a complementary strategy for identifying low-abundance miRNAs (Lai *et al.*, 2003; Bonnet *et al.*, 2004; Jones-Rhoades and Bartel, 2004; Wang *et al.*, 2004a; Adai *et al.*, 2005). The computational prediction of miRNAs is based on the properties of already-
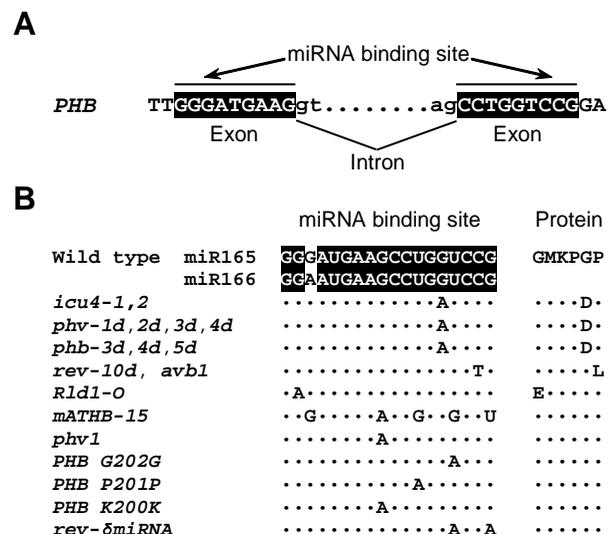
A

B

		miRNA binding site	Protein
Wild type	miR165	GGGAUGAAGCCUGGUCCG	GMKPGP
	miR166	GGAAUGAAGCCUGGUCCG	
icu4-1,2		·············A····	····D·
phv-1d,2d,3d,4d		·············A····	····D·
phb-3d,4d,5d		·············A····	····D·
rev-10d, avb1		················T·	·····L
Rld1-O		·A················	E·····
mATHB-15		··G·····A··G··G··U	······
phv1		·········A········	······
PHB G202G		·············A····	······
PHB P201P		···········A······	······
PHB K200K		·······A··········	······
rev-δmiRNA		·············A··A	······

Fig. 2. Nucleotide changes within the miR165/166 binding site of HD-Zip III genes. (A) *The miRNA binding site of the* PHB *gene.* **(B)** *Alleles and transgenes of HD-Zip III gene family members from* Arabidopsis thaliana, Zea mays (Rld1-O) and Nicotiana sylvestris (phv1) with mutations affecting the miR165/166 binding site. The sequences have been taken from Ochando* et al. *(submitted), McConnell* et al. *(2001), Emery* et al. *(2003), Zhong and Ye (2004), Juarez* et al. *(2004), Kim* et al. *(2005), McHale and Koning (2004) and Mallory* et al. *(2004a).*

known pre-miRNAs, including their preferential location within non-protein coding or intronic regions, their high conservation across related species, their ability to form stem-loop secondary structures and their characteristic pattern of nucleotide divergence (Lai *et al.*, 2003). The extraordinary sequence conservation of the stem-loops in related animal species, often higher than that of protein-coding genes, has been useful for miRNA prediction (Ambros *et al.*, 2003b; Lai *et al.*, 2003; Lim *et al.*, 2003a; 2003b). In plants, the variable length and higher complexity of the stem-loops makes the prediction of miRNAs more difficult than in animals (Llave *et al.*, 2002b; Reinhart *et al.*, 2002). To identify new miRNAs, most algorithms take advantage of the availability of the full genome sequences of rice and *Arabidopsis* (Bonnet *et al.*, 2004; Jones-Rhoades and Bartel, 2004; Wang *et al.*, 2004a; 2004b; Adai *et al.*, 2005). Often, the search for new miRNAs focuses on the pool of intergenic and intronic sequences of *Arabidopsis thaliana*, where most miRNAs are expected to be located (Bonnet *et al.*, 2004; Wang *et al.*, 2004a; Adai *et al.*, 2005). All approaches rely on the identification of sequences that meet the structural requirements of miRNAs and miRNA precursors, including properties such as the ability to form a stem-loop, the lengths of the predicted miRNA and the loop, the number of mismatches, or the G+C content (Bonnet *et al.*, 2004; Jones-Rhoades and Bartel, 2004; Wang *et al.*, 2004a; 2004b; Adai *et al.*, 2005). Some algorithms, which use the evolutionary conservation of miRNAs as a criterion for their predictions, also include rice sequences in their analyses (Bonnet *et al.*, 2004; Jones-Rhoades and Bartel, 2004; Wang *et al.*, 2004a; 2004b; Adai *et al.*, 2005). In some cases, these algorithms boost their predictive power by simultaneously searching for target mRNAs in the same genome (Adai *et al.*, 2005) or by searching for conserved mRNA:miRNA interactions in the genomes of *Arabidopsis* and rice (Jones-Rhoades and Bartel, 2004). The computational prediction of targets has been facilitated in plants by their near-perfect complementarity with the miRNAs (Rhoades *et al.*, 2002; Bonnet *et al.*, 2004; Jones-Rhoades and Bartel, 2004). Searches for mRNAs with three or fewer mismatches relative to the miRNA sequence have had a limited success in predicting targets in *Arabidopsis thaliana* (Rhoades *et al.*, 2002). By searching for conserved mRNA:miRNA interactions between *Arabidopsis* and rice, the method of Jones-Rhoades and Bartel was the most sensitive and allowed them to predict targets even when additional mismatches and gaps were present in the mRNA:miRNA duplexes (Jones-Rhoades and Bartel, 2004).

The miRNA Registry, a database aimed at standardizing the nomenclature of newly described miRNAs and at compiling the available information on published miRNAs, currently stores information on 112 *Arabidopsis*, 134 rice and 40 maize miRNAs genes (http://www.sanger.ac.uk/cgi-bin/Rfam/mirna/browse.pl; Griffiths-Jones, 2004).

Experimental validation of predicted miRNAs and their targets in plants

Several protocols based on the fact that most plant targets are downregulated by miRNA-directed cleavage are available for the validation of predicted miRNA targets. The Carrington lab pioneered this field by applying two experimental approaches to the study of the cleavage of suspected targets (Llave *et al.*, 2002a). The first approach uses a modified RNA ligase-mediated Rapid Amplification of cDNA Ends (5' RACE) protocol to map the cleavage site within the target mRNA taking advantage of the characteristically uncapped 5' phosphate groups of RNase III 3' products (Llave *et al.*, 2002b). This technique allowed them to validate, for the first time in plants, three *SCARECROW-LIKE (SCL)* genes as targets of miR39 (Llave *et al.*, 2002a).

The second approach involves the coexpression of constructs carrying a miRNA and its putative target in *Nicotiana benthamiana* leaves co-infiltrated with *Agrobacterium* (Llave *et al.*, 2002a). This technique has been applied to demonstrate the miR159-directed cleavage of *MYB33* (Achard *et al.*, 2004) and the miR39-directed cleavage of *SCL6* (Llave *et al.*, 2002a). By introducing point mutations that disrupt the complementarity of the miRNA with the target transcript, this method has also allowed researchers to study the importance of base pairing for cleavage (Llave *et al.*, 2002a).

To assess the role of cleavage in the repression of miRNA targets, several authors have engineered cleavage-resistant versions of the corresponding genes by introducing silent mutations that disrupt the complementarity of the miRNA and the mRNA. Plants expressing cleavage-resistant transgenes of *CUP-SHAPED COTYLEDON1* (*CUC1*), *CUC2*, *MYB33*, *TCP2*, *TCP4* and *PHB* displayed a range of mutant phenotypes that indicate the importance of miRNA regulation for plant development (Palatnik *et al.*, 2003; Laufs *et al.*, 2004; Mallory *et al.*, 2004a; 2004b; Millar and Gubler, 2005). In addition to these methods, evidence for the expression of computationally predicted miRNA genes comes from a variety of sources, including Northern blots, 5' RACE, ESTs and the identification of the predicted miRNA in the *Arabidopsis* Small RNA Project database or in libraries of small RNAs (Wang *et al.*, 2004a; Jones-Rhoades and Bartel, 2004; Gustafson *et al.*, 2005).

The evolution of miRNA genes

Little is known about the evolution of plant miRNAs. Many miRNA genes are conserved in rice and *Arabidopsis thaliana*, indicating that their origin predates the divergence of monocot and dicot plants approximately 150 million years ago (Pasquinelli *et al.*, 2000; Floyd and Bowman, 2004). The conservation of the miRNA binding sites of class III homeodomain-leucine zipper (HD-ZIP III) genes from bryophytes to seed plants indicates that the posttranscriptional regulation mediated by miR166 is a mechanism that can be traced back more than 400 million years (Floyd and Bowman, 2004). In contrast, a recent study has shown that some miRNA genes of *Arabidopsis thaliana* have originated recently and are not conserved in monocots and dicots (Allen *et al.*, 2004). These miRNAs seem to derive from inverted duplications and, because of their recent origin, do not belong to miRNA gene families (Allen *et al.*, 2004). According to the model of Allen and colleagues, the transcription of inverted duplications of target genes results in the formation of hairpins that are eventually processed into miRNAs by the miRNA machinery, leading to the targeting of the transcripts of the original gene (Allen *et al.*, 2004). Two miRNA genes of *Arabidopsis thaliana* not conserved in monocots are *MIR161* and *MIR163*, in which the similarity between the miRNA genes and their targets extends outside the miRNA, in both the miRNA and the miRNA* arms, as might be expected if their origin is recent (Allen *et al.*, 2004). In the same

way as miRNAs, *MIR161* and *MIR163* require *DCL1* and *HEN1* for their biogenesis and are able to direct the cleavage of their targets (Allen *et al.*, 2004).

The control of plant development by miRNAs: some examples

The targets of plant miRNAs often encode members of large families of transcription factors important for development, a finding consistent with the pleiotropic developmental defects caused by mutations in genes that encode elements of the miRNA pathway (Figure 1C-G; Table 1; Rhoades *et al.*, 2002; Dugas and Bartel, 2004; Mallory *et al.*, 2004a; 2004b). In addition, mutations in some target genes and in the miRNA genes that regulate them also cause developmental defects (Figure 1H). Below, we briefly summarize current knowledge on the function of some plant miRNAs, their targets and the developmental processes in which they are involved (Table 1).

miR-JAW

With the isolation of four independent activation-tagged alleles, *MIR-JAW* became the first miRNA gene to be identified in a mutant screen (Palatnik *et al.*, 2003). The pleiotropic phenotype of dominant *jaw-D* mutants resembled that of *cincinnata* (*cin*) mutants of *Antirrhinum majus* in the aberrant curling of the leaves, whose surface had a non-zero Gaussian curvature (Nath *et al.*, 2003; Palatnik *et al.*, 2003). *CIN* encodes a member of the TCP family of transcription factors (Nath *et al.*, 2003), named after its first characterized members: *teosinte branched1* (*tb1*) of maize, *cycloidea* (*cyc*) of *Antirrhinum majus* and *PCF1* and *PCF2* of rice (Cubas *et al.*, 1999). Members of this family are thought to regulate cell division and growth (Cubas *et al.*, 1999). This and the similarity of the *cin* loss-of-function and *jaw-D* gain-of-function phenotypes made TCP genes good candidates to be targets of miR-JAW. *MIR-JAW* was found to be expressed in wild-type shoot apices, inflorescences and siliques (Palatnik *et al.*, 2003).

The overexpression of *MIR-JAW* in *jaw-D* mutants caused cotyledon epinasty, fruit abnormalities, serration of leaf margins and delay in flowering (Palatnik *et al.*, 2003). Five TCP genes, closely related to *CIN*, were found to be downregulated in a microarray analysis of *jaw-D*, suggesting that they were targets of miR-JAW (Table 1; Palatnik *et al.*, 2003). Sequence alignment of these TCP genes uncovered a conserved nucleotide sequence that was also present at the *MIR-JAW* locus. To confirm the negative regulation guided by miR-JAW, Palatnik *et al.* (2003) mapped the cleavage sites using modified 5' RACE and created cleavage-resistant versions of *TCP2* and *TCP4* by introducing silent mutations in the miR-JAW binding site. The effects of expressing these modified *TCP2* or *TCP4* genes in transgenic plants were more dramatic for *TCP4* than for *TCP2*, the former determining organ fusions and the absence of the shoot apical meristem (Palatnik *et al.*, 2003). All these results indicate that, by restricting the expression of TCP genes, miR-JAW regulates not only the development of leaves but also many other aspects of plant development (Palatnik *et al.*, 2003).

miR159

Despite its sequence similarity to miR-JAW, miR159 seems to regulate a non-overlapping set of targets that includes the AtGAMYB

genes *MYB33* and *MYB65* (Table 1; Palatnik *et al.*, 2003). Both *MYB33* and *MYB65* contain sequences that allow miRNA regulation by miR159 (Palatnik *et al.*, 2003). These genes code for MYB transcription factors related to barley GAMYB, a gibberellin-responsive transcriptional regulator that activates other gibberellin-regulated genes (Gubler *et al.*, 1999). The levels of miR159 also depend on gibberellic acid (GA), as inferred from their decrease in GA-deficient *ga1* mutants or their increase after the application of exogenous GA (Achard *et al.*, 2004).

Transgenic plants overexpressing miR159 showed several recognizable phenotypes. First, the transition to flowering was delayed under short day conditions, a phenotype attributable to decreased transcript levels of the AtGAMYB gene *MYB33* and its putative target, the floral meristem identity gene *LEAFY* (Achard *et al.*, 2004). Second, these plants were not fertile due to defective anther development (Achard *et al.*, 2004), a phenotype also observed in *myb33 myb65* double mutants (Millar and Gubler, 2005). The anther phenotype was conditionally restored under high-light or low-temperature conditions, indicating that the functionally redundant *MYB33* and *MYB65* genes are not fully essential for anther development (Millar and Gubler, 2005). Consistent with the phenotype, a translational *MYB33:GUS* fusion was expressed in the anthers (Millar and Gubler, 2005). A number of observations support the importance of miR159-mediated restriction of the spatial expression pattern of *MYB33* in plant growth and development. The expression pattern expanded to tissues outside the anther in plants carrying a cleavage-resistant version of the *MYB33::GUS* fusion, indicating the importance of miR159 for confining the expression to the anthers (Millar and Gubler, 2005). Palatnik *et al.* (2003) had previously reported that most plants expressing a miRNA-resistant version of *MYB33* developed curled-up leaves, which indicates that the downregulation of *MYB33* is important for normal leaf development. Besides leaf incurvature, the cleavage-resistant *MYB33::GUS* fusion conferred additional phenotypes, including rounded leaves with short petioles, a failure to expand the cotyledons, a small size, reduced apical dominance and low fertility (Millar and Gubler, 2005), suggesting that miR159 plays an important regulatory role in plant development.

miR165 and miR166

Multiple mutant combinations have shown that the five HD-ZIP III genes of *Arabidopsis thaliana* redundantly regulate important aspects of development, including the patterning of vasculature, the establishment or maintenance of abaxial-adaxial polarity in lateral organs and the function of meristems (Emery *et al.*, 2003; Hawker and Bowman, 2004; Prigge *et al.*, 2005). All the semidominant, gain-of-function alleles of four of them, *PHB*, *PHV*, *REVOLUTA* (*REV*) and *INCURVATA4* (*ICU4*; Serrano-Cartagena *et al.*, 2000), the latter also known as *ATHB-15* and *CORONA (CNA)*, damage a complementary site for miR165 and miR166 located in the START domain characteristic of HD-ZIP III proteins (Figure 2B) and confer pleiotropic phenotypes affecting the processes mentioned above (Figure 1H; McConnell and Barton, 1998; McConnell *et al.*, 2001; Eshed *et al.*, 2001; Emery *et al.*, 2003; Juarez *et al.*, 2004; McHale and Koning, 2004; Zhong and Ye, 2004; Green *et al.*, 2005; Kim *et al.*, 2005; Prigge *et al.*, 2005; Ochando *et al.*, submitted).

The miR165 and miR166 miRNAs are very similar in sequence and derive from multiple loci in the genome (Reinhart *et al.*, 2002).

Evidence supporting the miRNA-guided cleavage of *REV*, *PHB*, *PHV*, *ATHB-8* and *ICU4* (*ATHB-15* and *CNA*) comes from different sources. First, the cleaved products have been identified using modified 5' RACE (Emery *et al.*, 2003; Mallory *et al.*, 2004a; Kim *et al.*, 2005). Second, HD-ZIP III genes are downregulated by an activation tagged allele of miR166a, *men1*, as is to be expected if they are targets of miR166 (Kim *et al.*, 2005). Third, the introduction of cleavage-resistant versions of *REV* (*rev-δmiRNA;* Figure 2B) and of *PHB* (*PHB G202G; PHB P201P; PHB K200K;* Figure 2B) in wild-type plants phenocopied the effects of gain-function *rev* and *phb* mutations (Emery *et al.*, 2003; Mallory *et al.*, 2004a). Fourth, we have found synergistic phenotypes in double mutants involving the gain-of-function *icu4-1* mutation (which damages its miR165/166 binding site; Figures 1H, 2B) and loss-of-function alleles of genes of the miRNA pathway, such as *AGO1* or *HYL1* (Jover-Gil *et al.*, submitted; Figure 1M, N). Lastly, the study of dominant alleles of *PHB* and *PHV* has led to a model of transcriptional repression in *cis* guided by miRNAs (see 'Transcriptional regulation by miRNAs' above; Bao *et al.*, 2004).

miR164

The cleavage of several NAC-domain genes is mediated by miR164, including *CUC1*, *CUC2* and *NAC1* (Table 1; Laufs *et al.*, 2004; Mallory *et al.*, 2004b; Baker *et al.*, 2005). Reflecting this, plants overexpressing *MIR164a* and *MIR164b* showed cotyledon and floral organ fusions similar to those of *cuc1 cuc2* double mutants (Aida *et al.*, 1997; Laufs *et al.*, 2004; Mallory *et al.*, 2004b) and had reduced levels of *CUC1* and *CUC2* transcripts (Laufs *et al.*, 2004). It has been proposed that the leaf and stem fusions observed in these plants, but not observed in *cuc1 cuc2* double mutants, arise from the downregulation of other target genes, such as At5g07680, At5g61430 or *NAC1* (Laufs *et al.*, 2004; Mallory *et al.*, 2004b).

The transcripts of *CUC1*, *CUC2* and *NAC1* are cleaved at the miR164 binding site in wild-type plants (Mallory *et al.*, 2004b). The abnormal embryonic, vegetative and floral development and a boundary enlargement phenotype caused by miR164-resistant versions of *CUC1* and *CUC2* supports a role for miR164 in the control of boundary size in meristems and the formation and separation of embryonic, vegetative and floral organs (Laufs *et al.*, 2004; Mallory *et al.*, 2004b). The absence of a mutant phenotype in a null *MIR164b* mutant points to overlapping functions with other *MIR164* genes, such as *MIR164a* (Mallory *et al.*, 2004b). The *early extra petals1 (eep1)* mutant led to the identification of a third locus for this miRNA, *MIR164c* (Table 1; Baker *et al.*, 2005). Loss of function of *MIR164c* causes an increase in the number of petals, demonstrating that at least some members of a miRNA family play specific functions that can be uncovered through mutational analysis (Baker *et al.*, 2005).

miR172

Several members of the APETALA2 (AP2) family are predicted targets of miR172, including the floral homeotic gene *AP2* and the *TARGET OF EAT1* (*TOE1*), *TOE2* and *TOE3* genes in *Arabidopsis thaliana* and *INDETERMINATE SPIKE-LET1* and *GLOSSY15* in *Zea mays* (Table 1; Park *et al.*, 2002; Aukerman and Sakai, 2003). So far, miR172 is the only known plant miRNA that acts through translational repression, as most

animal miRNAs, rather than the cleavage of target transcripts (Aukerman and Sakai, 2003; Chen, 2004). An activation tagged allele of one of the five *MIR172* loci present in the genome of *Arabidopsis thaliana*, named *early activation tagged (eat-D)*, causes the overexpression of miR172a-2 and the translational repression of *AP2* (Aukerman and Sakai, 2003). Accordingly, the phenotype of *eat-D* plants resembled that of *ap2* mutants and included early flowering, missing petals and the homeotic conversion of sepals into carpels. While a *35S-EAT* transgene conditioned the same phenotype as *eat-D* mutants, another one with a deletion of the 21-nucleotide miRNA had no effect, demonstrating that the overexpression of the miRNA was responsible for the mutant phenotype (Aukerman and Sakai, 2003). In the same screen, Aukerman and Sakai also found an activation-tagged allele of the miR172 target gene *TOE1*. The overexpression of *TOE1* in the *toe1-1d* mutant caused delayed flowering. On the contrary, loss-of-function alleles of *TOE1* and *TOE2* caused early flowering, which indicates that these genes behave as flowering repressors. These results are consistent with the *MIR172* genes functioning to control the flowering time and the identity of floral organs (Aukerman and Sakai, 2003; Chen, 2004).

Perspectives

The discovery of RNAi and the identification of *let-7* stimulated searches for miRNAs and siRNAs in animals and plants, as well as searches for the proteins involved in these silencing phenomena (Elbashir *et al.,* 2001; Lagos-Quintana *et al.*, 2001). Some estimates indicate that about 1% of the genes in animal genomes code for miRNAs (Lai *et al.*, 2003; Lim *et al.*, 2003a; 2003b) and that more than 20% of the human genes may be controlled by miRNAs (Lewis *et al.*, 2005; Xie *et al.*, 2005). In *Arabidopsis thaliana*, an increasing number of miRNAs have been found to be involved in the regulation of developmental processes, such as the transition to flowering, the function of meristems, the morphogenesis of leaves, the patterning of the vasculature or the development of flowers. The availability of genomic sequences for additional plant species (maize, poplar, rice, sorghum), as well as of improved protocols and computational algorithms for miRNA identification, will help to increase the pool of known miRNA genes in plant genomes. With the identification of new targets, we will also gain insight on the developmental pathways regulated by miRNAs.

Our knowledge of the miRNA biogenesis is growing quickly, as shown by recent reports on new components of the pathway and on their functions in plants and animals. Additional components will certainly be identified using biochemical and genetic techniques. Among these, mutational screens may take advantage of already-known components of the miRNA pathway by searching for enhancers or suppressors of the phenotypes associated to these components. A better understanding on miRNA biogenesis, regulation and function will open the door to the design of artificial miRNAs that may be used as efficient tools for controlling gene expression at will.

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