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The developmental role of microRNA in plants

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MicroRNAs (miRNAs) are single-stranded RNA molecules of around 22 nucleotides (nt) in length that are associated with the RNA-induced silencing complex (RISC). They play an important role in plant development, either by targeting mRNA for cleavage or by inhibiting translation. Over the past year, the list of known miRNAs, confirmed targets and developmental effects has expanded, as has the realization that they are conserved during evolution and that small RNAs can play a direct role in cell–cell signaling.

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Abbreviations

AGO	ARGONAUTE
AP2	*APETALA2*
ARF	auxin response factor
AtXRN4	*Arabidopsis thaliana* EXORIBONUCLEASE4
CUC	*CUP-SHAPED COTYLEDONS*
DCL3	*DICER-LIKE3*
dsRNA	double-stranded RNA
GA	gibberellic acid
HEN1	HUA-ENHANCER1
HST	HASTY
HYL1	HYPONASTIC LEAVES1
miRNA	microRNA
PAZ domain	PIWI/AGO1/ZWILLE domain
PHB	*PHABULOSA*
PHV	*PHAVOLUTA*
PNH	*PINHEAD*
PTGS	post-transcriptional gene silencing
REV	*REVOLUTA*
RISC	RNA-induced silencing complex
SDE1	SILENCING DEFECTIVE1
siRNA	small interfering RNA
TIR1	TRANSPORT INHIBITOR RESPONSE1
ZLL	*ZWILLE*

Introduction

Small interfering RNAs (siRNAs) are derived from transposon, transgene or viral double-stranded RNA (dsRNA) duplexes. They are found in most eukaryotes, and are the most abundant small RNA in plants [1–4]. MicroRNAs (miRNAs), on the other hand, are derived from hairpin precursors (pre-miRNA) from which both miRNA and the imperfectly complementary miRNA* strands are released. miRNA sequences are not conserved between animals and plants, and none have been found in fungi. Within kingdoms, many miRNAs have an ancient origin, some being perfectly conserved among *Arabidopsis*, rice and even mosses, liverworts and hornworts [5,6••,7•,8•,9••]. Plant miRNAs often match their target mRNAs closely [5], and target sequences have also been conserved so that it is possible to identify miRNA genes computationally by searching for conserved hairpins and target sequences [6••,8•,10]. This approach has revealed 92 loci in *Arabidopsis* [6••,8•] that encode 27 distinct miRNAs, and a similar number in rice (Table 1).

Biogenesis of microRNAs and assembly into RISC

miRNA loci encode transcripts of approximately 1 kb termed pri-miRNA, which are capped and polyadenylated and appear as non-coding expressed sequence tags (ESTs) [11••]. Transgenic experiments indicate that it is the structure rather than the sequence of the pre-miRNA that directs their correct processing and that the rest of the pri-miRNA is not required for the production of miRNAs [12••,13]. pri-miRNA sequences are not conserved between family members, but may be involved in differential regulation. DICER cleaves dsRNA to generate siRNA during RNA interference [14]. In *Caenorhabditis elegans*, the same Dicer is required for miRNA, but in *Drosophila*, Dicer-1 and Dicer-2 have these distinct functions [15]. There are four Dicer-like genes in *Arabidopsis* that each encode a PIWI/AGO1/ZWILLE (PAZ) domain, a DExH-box RNA helicase-C, two ribonuclease III domains and at least one dsRNA-binding domain (except for *DICER-LIKE3* [*DCL3*]). DCL1, DCL2 and DCL3 are required for the biogenesis of miRNA, viral siRNA and transposon siRNA, respectively [16••]. In animals, the pri-miRNA is cleaved in the nucleus by the DICER family member Drosha to produce a 60–70 nt pre-miRNA [13]. Drosha does not exist in plants, but DCL1 has a nuclear localization signal, suggesting it may process the pri-miRNA as well as the pre-miRNA [3,4,16••,17,18]. miRNA biogenesis in plants also requires HUA ENHANCER1 (HEN1) [4,19,20•], which has two dsRNA-binding domains and a nuclear localization signal [4]. HEN1 is conserved in fungi and, unlike DCL1, is also required for post-transcriptional gene silencing (PTGS) [4,19,21]. Redundancy with a closely linked homolog may alleviate *hen1* phenotypes relative to *dcl1* [4,19] (Figure 1).

Table 1

Role and conservation of *Arabidopsis* miRNA.

Role	miRNA in *Arabidopsis*	Target genes	Conserved in	Reference(s)
Regulation of miRNA	162	*DCL1*	Rice	[20•]
	168	*AGO1*	Rice	[40•]
Hormone response	159	*GA-MYB*	Moss	[69]
	160	ARFs	Gymnosperms	
	167	*ARF8*	Gymnosperms	
	164	*NAC1*	Rice	
	393	F-box proteins, bHLH transcription factors	Gymnosperms	
Patterning	164	*CUC1*, *CUC2*	Rice	[45•,61••]
	165	*PHB*, *PHV*, *REV*		[31••,32••,62••,63••]
	166	*PHB*, *PHV*, *REV*	Hornwort[(a)]	[30••–32••,62••,63••,74]
Control of cell division	JAW	*TCP2*, *TCP3*, *TCP4*, *TCP10*, *TCP24*	Moss	[42••]
Flowering	156	*SPBL2*, *SPBL10*		[65•]
	EAT/172	*AP2*, *TOE* (translation)		[11••,41••]
Other transcription factors	169	*CCAAT-binding factor, HAP2-like*		
	396	Growth-regulating factors	Gymnosperms	
	171	GRAS domain (*SCR*)	Fern	
Environmental/stress responses	398	Copper superoxide dismutases,	Gymnosperms	
		Cytochrome C oxidase subunit V	Gymnosperms	
	395	ATP sulfurylases	Gymnosperms	[6••]
Others	399	Phosphate transporter		
	161	PPR repeat		
	394	F-box protein genes		
	158	Hypothetical **At1 g64100**		
	163	Hypothetical **At1 g66700**, **At1 g66690**		
	173	Hypothetical **At3 g28460**		
	397	Laccases, Beta-6-tubulin	Gymnosperms	
	174	Hypothetical **At1 g17050**		
	175	Hypothetical **At5 g18040**, **At3 g43200**, **At1 g51670**		

[(a)]Cleavage shown Floyd and Bowman [9••].

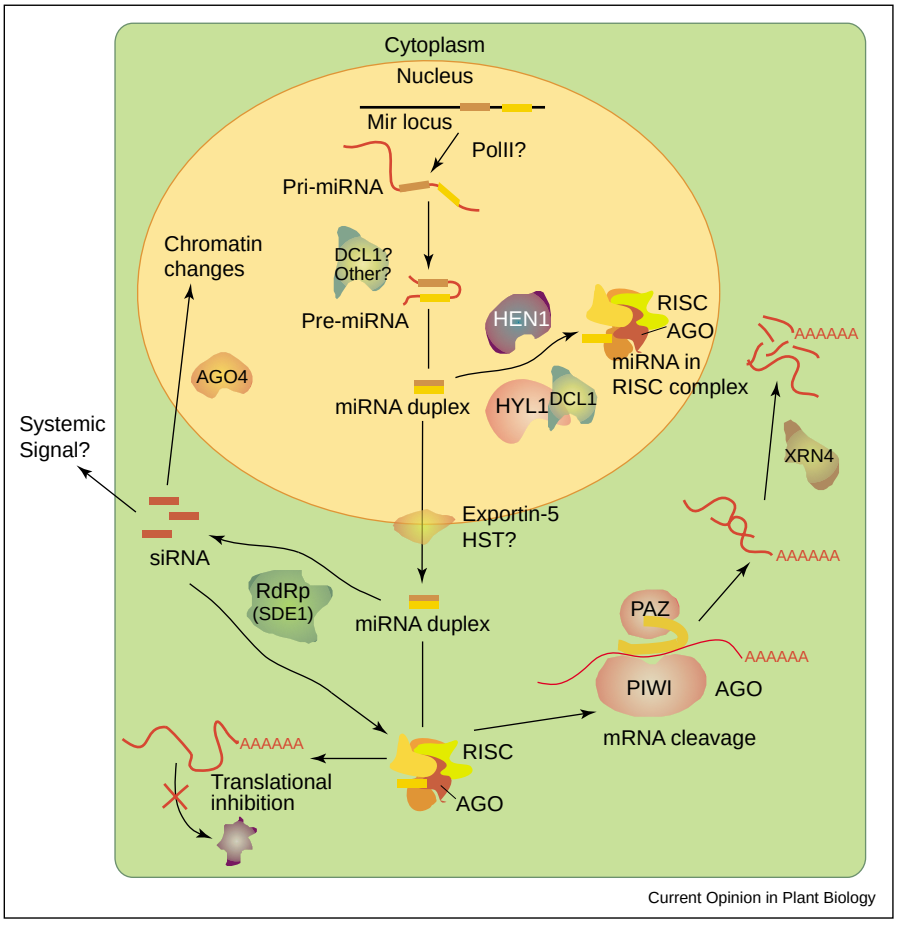
In animals, the miRNA–miRNA* duplex is transported out of the nucleus by Exportin-5 [22–24]. This duplex is then unwound in the cytoplasm by an unidentified helicase and taken up by the RNA-induced silencing complex (RISC)-loading complex, which includes the protein R2D2 [25•]. Plants have an Exportin-5 homolog, HASTY (HST) [26], as well as an R2D2 homolog HYPONASTIC LEAVES1 (HYL1) [27–29]. miRNA processing has not been examined in the *hst* background but a subset of miRNA are reduced in *hyl1*, as is the cleavage of a target mRNA. Like DCL1, HYL1 is not required for PTGS [28,29], has two dsRNA-binding domains, and preferentially binds dsRNA *in vitro* [27]. Unlike animal R2D2, however, HYL1 is nuclear localized in rings and small bodies [29]. The rarity of cloned miRNA suggests that the miRNA–miRNA duplex is short-lived [3]. Both the pri-miRNA and pre-miRNA can only be detected by RT–PCR, indicating their low abundance [30••–32••]. The relatively high levels of miRNA indicate that processing of these precursors is swift, and may occur in the nucleus for at least some miRNA, explaining the mild developmental phenotypes of *hst* compared to those of *dcl1* [33].

Mechanism

RISCs all comprise an ARGONAUTE (AGO) protein and either miRNA or siRNA, as well as other proteins that are specific to each complex [25•]. AGO proteins are defined by the presence of a PAZ domain (also found in Dicers) and a PIWI domain [34], both of whose structures are known. A hydrophilic cleft in the PAZ domain binds the 3′ end of single-stranded RNA molecules [25•]. The PIWI domain has a structure similar to that of RNase H, and mutations in the PIWI domain of mouse Ago2 destroy RISC activity [35••]. A highly conserved histidine residue near the active site of the PIWI domain is mutated to leucine in the *Arabidopsis ago1-12* allele, providing further evidence of the functional importance of this domain [32••,36•]. It is thought that the 3′ end of the miRNA is held in a groove in the PAZ domain, and aligns with the target mRNA that is held in a groove in the PIWI domain. The phosphate between the 11th and 12th residues of the miRNA falls near the active DDE site and is cleaved.

Ten *AGO*-like genes are found in *Arabidopsis*, with even more in rice. *AGO1* itself is required for PTGS, but the closely related gene *PINHEAD* (*PHN*)/*ZWILLE* (*ZLL*) is

Figure 1



Model for miRNA biogenesis and activity in plants. Transcription of miR loci by PolII-like activity generates a pri-miRNA of about 1 kb. This pri-miRNA is processed by DCL1 and possibly other proteins to yield the fold-back precursor pre-miRNA. The pre-miRNA may be trafficked out of the nucleus by HST (Exportin-5) or it may be retained in the nucleus, where HEN1, HYL1 and DCL1 are localized. These proteins process the pre-miRNA further to form a miRNA–miRNA imperfect duplex, which is unwound and loaded on to the RISC (HYL1 and DCL1 may act together here). The RISC includes an AGO protein, which binds the 3′ miRNA overhang in the PAZ domain. The RISC complex is guided by the miRNA to the target mRNA, possibly by a helicase scanning mechanism (not shown). The target lies along the cleft in the PIWI domain of the mRNA, and is cleaved between the 10th and 11th bases from the 5′ end of the miRNA match. The cleaved halves of the target mRNA are then degraded by XRN4, as well as by secondary siRNA that arise from the action of RNA-dependent RNA polymerase (RdRP), most probably in the cytoplasm. The RISC can also mediate translational inhibition by an unknown mechanism. AGO complexes (such as AGO4) target heterochromatin formation via siRNA.

not, although it may be redundant. AGO4 silences some transposons and repeats [37], as does AGO1 [38], but ZIPPY/AGO7 appears to have effects only on development [39•]. AGO1 might be required for the accumulation of some miRNAs, but this is not a general role in biogenesis as other miRNAs overaccumulate in the absence of AGO1 ([32••,40•]; M Ronemus, M Vaughn, R Martienssen, unpublished). Instead, AGO1 may stabilize miRNA in some cases but consume it in others by affecting downstream mechanisms, leading to the accumulation of miRNAs in mutants. Both *DCL1* and *AGO1* are themselves regulated by miRNAs [20•,40•].

The effect of a miRNA on a target mRNA depends on the match between them. In animals, mis-matches in the middle of the miRNA lead to translational inhibition rather than to cleavage [25•], but in plants, this is not the case. miR172 matches *APETALA2* (*AP2*) and *AP2*-like genes almost perfectly, but causes translational repression as well as cleavage [11••,41••]. In most cases, however, cleavage of the mRNA at a site half way along the miRNA match is the predominant mechanism in plants [42••,43,44]. Experiments using artificial targets of miR171 [12••] and *PHABULOSA* (*PHB*) transgenes [45•] indicate that intact base pairing in the center and at the 5′ side of the match is required for target cleavage. A survey of the matches between known miRNA–mRNA pairs that lead to cleavage showed that exact matches were common at nucleotides 3–10 and rarer at the very ends of the sequences, especially at the 3′ ends [45•]. This

is similar to the situation in animals and might be related to the orientation of miRNA in the RISC and the spatial dynamics of pairing [25•].

Following mRNA cleavage, the miRNA is unchanged and can guide the RISC to further targets [46•], but the mRNA cleavage products are degraded, starting from the cleavage site. The exosome may degrade the 5′ segment [47], whereas in many cases, 5′ to 3′ degradation of the 3′ end of cleaved mRNA is accomplished by AtXRN4 (*Arabidopsis thaliana* EXORIBONUCLEASE4), a homolog of the yeast Xrn1p [48]. Other cleaved transcripts are unaffected in *xrn4* mutants, suggesting that a second pathway may exist. miRNA cleavage also leads to the generation of siRNA from sequences that are adjacent to the miRNA match site ([12••]; M Ronemus, M Vaughn, R Martienssen, unpublished). Formation of these siRNAs requires the RNA-dependent RNA polymerase SILENCING DEFECTIVE1 (SDE1) ([12••]; M Ronemus, M Vaughn, R Martienssen, unpublished). *sde1* mutants have mild phenotypes that resemble those of *hst* mutants, and it is tempting to speculate that siRNA production requires exportin whereas miRNA production does not. miRNA-regulated genes that also generate siRNA would be expected to be recessive, rather than dominant, when mutated at the miRNA site because siRNA from the wildtype allele could still silence the mutant alleles; no such mutations have been described to date.

RNA interference that is mediated by siRNA is transmissible in grafts [49]. In an *sde1* background, the silencing of green fluorescent protein (GFP) fusions mediated by endogenous miR171 was limited to cells in which miR171 was expressed, whereas in wildtype plants, this silencing was much more extensive [12••]. This evidence suggests that non-cell-autonomous effects of miRNA regulation are mediated by siRNAs. However, miRNAs have been found in cucurbit phloem [50••], including miR171, miR156, miR159 and miR167. These miRNAs cannot traffic alone, their trafficking requires the protein CmPSRP1 (*Curcurbita maxima* PHLOEM SMALL RNA-BINDING PROTEIN1), which selectively binds 25 nt ssRNA. PSRP1 is found in *Curcurbita maxima* (pumpkin), *Cucumis sativus* (cucumber) and *Lupinus alba* (lupin) but is not conserved in *Arabidopsis* or rice.

A pleiotropic role for miRNA in development

Mutations in *AGO1* that result in the loss of both the PAZ and the PIWI domains produce organs that are radial, plants that are sterile, and seedlings that germinate but often lose meristem function [32••,51–53]. Mutants in which only the PIWI domain is affected have weaker phenotypes, with adaxialized organs and recognizable flowers [32••], and are fertile in some backgrounds [53]. Mutations in PNH/ZLL, which is closely related to AGO1, reveal that PNH/ZLL has developmental roles that overlap with AGO1 function [52]. PNH/ZLL is required for post embryonic development of the shoot apical meristem and for axis determinacy, but *pnh/zll* plants do not show polarity defects in lateral organs as *ago1* mutants do [52,54–56]. The role of PNH/ZLL is retained in rice, where it has a role in vascular development [57]. The effects of *zippy* on developmental timing are subtle, and resemble those of *sde1* [39•], indicating that, like SDE1, ZIPPY acts in a pathway that involves siRNA.

Different alleles of *dcl1* have a range of phenotypes that are reflected by *dcl1*'s synonyms: *embryo lethal-76*, *suspensor defective*, *carpel factory*, and *short integuments* (*sin*) [33]. A role for miRNA in signaling from maternal tissues to the developing embryo is suggested by the phenotypes of *dcl1-sin1*. Alternatively, these phenotypes might be related to a role for miRNA in imprinting, as reporter-gene expression suggests that *DCL1* is paternally imprinted [58]. *hen1* and *hyl1* mutants resemble weak *dcl1* mutants. *hen1* was originally isolated as an enhancer of *hua1*, a regulator of floral organ identity, but is also allelic to the inflorescence mutant *corymbosa-2* [4,21]. *hyl1* affects leaf development, apical dominance and hormone sensitivity. It has increased sensitivity to abscisic acid and lowered sensitivity to cytokinin and auxin [27–29]. Complete loss of *HEN1* and *HYL1* function causes phenotypes that are much less severe than those of *ago1* or *dcl1* mutants, indicating that *HEN1* and *HYL1* are redundant with other genes for miRNA biogenesis and function.

These phenotypes suggest that miRNAs are required for meristem function, organ polarity and vascular development, floral patterning and hormone response. These roles have been confirmed by sequencing miRNAs (Table 1), many of which are developmentally or environmentally regulated [6••,7•]. In all, 83 target genes have been predicted and 48 miRNA–target interactions have been confirmed by cleavage assays [6••]. Although several targets have unknown functions, others are known to contribute to various aspects of development and gene regulation (Table 1).

A large proportion of transcription factors have been identified as miRNA targets (nearly half), and it has been proposed that miRNAs have a role in 'clearing out' regulatory genes when a cell's fate is changed [25•]. For example, *CUP-SHAPED COTYLEDONS* (*CUC*) genes are required to specify organ boundaries and to positively regulate *SHOOTMERISTEMLESS1* (*STM1*) [59,60]. CUC1 and CUC2 are redundant factors, and are both regulated by miR164 [61••,62••]. The HD-ZIP III genes *PHB*, *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*), and their regulation by miRNA, are highly conserved within green plants [9••]. In both monocots and dicots, regulation by miR165/mi166 is required for organ axis specification, for vascular development and for meristem function [30••–32••,45•,63••]. Analysis of the phenotypes caused by mutations in these genes suggests they

may have a general role in the control of indeterminate cell fate [31••].

miRJAW is also required for the control of leaf development. However, this miRNA regulates cell division in the expanding *Arabidopsis* leaf via a subset of TCP genes, which promote the transcription of genes that are involved in DNA replication [43]. In *Antirrhinum*, the expression of *CINCINNATA*, a TCP gene, marks the advancing boundary of cell division arrest [64]. Transitions in the plant life cycle are also regulated by miRNA. miR172 is required for the regulation of AP2 and AP2-like proteins, which are required for flowering and floral organ identity [11••,41••]. miR156 is upregulated upon flowering and is required for the regulation of several members of the *SQUAMOSA PROMOTER BINDING LIKE* (*SPL*) family (M Ronemus, M Vaughn, R Martienssen, unpublished), which suppress flowering in *Arabidopsis* [65•] and promote epidermal cell fate in maize [66].

miRNA regulation is not limited to patterning and cell cycle control, but also has a role in environmental and hormonal response [6••]. A major functionally related group of miRNA targets are those involved in hormone signaling, principally in signaling by auxin. Two sets of auxin response factor (ARF) genes are targets of miR167 and miR160, whereas TRANSPORT INHIBITOR RESPONSE1 (TIR1) is a target of mi393 and is conserved among rice, *Arabidopsis*, poplar, *Medicago* and *Lotus* [8•]. TIR1 binds Aux/indole-3-acetic acid (IAA) proteins in the E3 ubiquitin ligase SCF complex, which destroys them [67]. NAC1 (an miR164 target) acts downstream of TIR1 in the control of lateral root growth [68]. Finally, miR159 is regulated positively by gibberellic acid (GA) and negatively by the DELLA proteins, which are GA-response inhibitors [69]. miR159 regulates flowering time and anther development through cleavage of the mRNA of *GAMYB*, a positive regulator of *LEAFY*. There may also be feedback from GAMYB to regulation of the miRNA. This pathway is conserved in barley. The DELLA proteins also mediate auxin and ethylene responses, this regulatory loop may co-ordinate several hormone signaling pathways [70]. Finally, miR395 targets ATP sulfurylases and is itself regulated by sulfates, indicating that environmental responses can also be mediated by miRNA [6••].

Conclusions

Like transcriptional regulation, regulation of gene activity by miRNAs is not limited to any particular pathway. Rather, the uniqueness of miRNA regulation may lie in the redundancy of miRNA targets and in its feedback control. Feedback control allows miRNAs to act as rheostats of gene expression [71•]. This is particularly important in plants as a means of controlling redundant dose-sensitive genes following polyploidy [72]. miRNAs are not dose-sensitive and would prevent the duplication of transcription factors from causing a hugely amplified response. Another means of feedback control of miRNA regulation is by chromatin regulation. AGO and DICER are required for heterochromatin formation at *Schizosaccharomyces pombe* centromeres [73], and may play a role in epigenetic silencing [72]. The possibilities of controlling miRNA trafficking, either directly or via siRNA, are also intriguing. Alterations in the spatial distribution of miRNA in *ago1* indicate that AGO1 is involved in this process, either directly or indirectly [32••]. An attractive model is that small RNA acts as a signal during early development, which is remembered in later development through RNAi-dependent epigenetic mechanisms, such as heterochromatin formation. It remains to be seen how widespread such a mechanism will prove to be.

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