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Plant miRNA integrated functions in development and reproduction

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Plant development and reproduction are complex processes during which an individual fulfills its life cycle, starting from germination and the elaboration of new organs and growth, leading to the formation of reproductive structures and ultimately terminating in the production of the next generation. These mechanisms are the result of a long evolutionary history that has led to sophisticated regulatory mechanisms involving multiple levels of regulators. MicroRNAs (miRNAs) are a class of small regulatory molecules that play a pivotal role in regulatory networks by negatively controlling target genes. Since miRNA very first identification twenty years ago, they have attracted much interest for their role as essential regulators of plant development. In this review, we propose a comprehensive and critical analysis of the importance of miRNAs during plant development and reproduction. We begin by presenting the current understanding of miRNAs' evolutionary history, biogenesis, mode of action, position in regulatory networks, and their potential as mobile molecules, exploring how these aspects contribute to their functions in plant development and reproduction. Then, we explore the genetic strategies employed to effectively analyze their roles, with an emphasis on recent advancements resulting from genome editing techniques. Next, we focus on miRNA contributions to four crucial processes: growth, organ patterning and identity, life cycle progression and reproduction. Through this analysis, the importance of miRNAs during plant development and reproduction emerges, which we finally discuss in light of the current view miRNAs' roles during animal development.

KEYWORDS

miRNA, development, morphogenesis, growth, reproduction, robustness, gene regulatory network, mobile signal

1 Introduction

MicroRNAs (miRNAs) are small, single-stranded regulatory RNAs that repress target gene expression. It is generally assumed that they have evolved independently several times within eukaryotes (Tarver et al., 2012), though the opposite view has also been proposed (Moran et al., 2017; Tripathi et al., 2022). Since their identification in plants more than twenty years ago (Llave et al., 2002; Reinhart et al., 2002), miRNAs have been identified in

many plant species and appear to form a diverse genetic tool set encoded by multiple genes. For instance, 150 high-confidence *MIRNA* genes coding for 26 miRNA families have been identified in maize (Zhang et al., 2009). The observation that the first identified miRNAs could target conserved transcription factors playing key roles during plant development quickly promoted these molecules as important regulators of plant development (Rhoades et al., 2002; Floyd and Bowman, 2004). This perspective was further strengthened by the observation that key players in the biogenesis and function of plant miRNAs had previously been identified during developmental genetic screens (Garcia, 2008). Whereas it is clear that miRNAs as a whole are essential for plants because strong loss-of-function mutants in miRNA maturation machinery components display embryo lethal phenotypes (Schauer et al., 2002), the role of individual miRNAs is far less understood. In fact, in plants, like in other eukaryotes the importance of miRNAs during development is still a matter of debate. In this review, we will delve into the contribution of miRNAs to plant development. Our objective is not to present an exhaustive list of their roles but rather to explore how our current understanding of their evolution, biogenesis, and integration into regulatory networks sheds light on their significance in development. We will concentrate on recent genetic analyses that have exploded over the past five years through the use of genome editing techniques. These methodologies enable the systematic knockout of all members of MIRNA gene families, allowing to genetically define both their specific and redundant roles in plant development and reproduction. The roles of miRNAs that thus emerges in plants will be compared with the roles of miRNAs during animal development.

2 The miRNA repertoire of a plant is the result of its evolutionary history.

One criterion to assess the importance of miRNAs in plant development is to look at their evolutionary history and conservation. An expanding wealth of miRNA sequences is available for many species, which can be however obscured by the difficulty in distinguishing *bona fide* miRNA from other small RNAs (Taylor et al., 2014). A phylogenomic framework was used to attempt to reconstruct miRNA families evolution, which suggested that plant current miRNAs are in an important part inherited from the ancestral embryophyte and spermaphyte (Taylor et al., 2014). These studies showed that miRNAs root deep into the plant phylogeny, although their exact origin is still unknown as no shared miRNAs were found between the unicellular green algae *Chlamydomonas reinhardtii* and land plants (Nozawa et al., 2012). In addition to such ancient miRNAs, a large expansion of the miRNA families may have also occurred after the divergence of the flowering plants. To support evolution of the miRNAs, several mechanisms of miRNA gene formation have been proposed (Cui et al., 2017). As a result of such evolution, the miRNA repertoire of each plant species is formed by a continuum of ancient to younger miRNAs. The former ones are highly conserved, whereas the latter ones are specific to a genus, species, or even a particular accession (Figure 1). *MIRNA* genes often form families, with conserved miRNAs tending to form larger families and being on average expressed at higher levels than non-conserved ones (Figure 1; Rajagopalan et al., 2006; Fahlgren et al., 2007). An important hint pointing to the importance of miRNAs in plant development was the observation that conserved miRNAs tend to preferentially target transcription factors with well-established roles during plant development. Furthermore, this targeting was found to be evolutionary conserved (Rhoades et al., 2002; Floyd and Bowman, 2004). In contrast, targets of non-conserved miRNAs code for proteins with much more diverse biological functions (Fahlgren et al., 2007). In summary, miRNAs constitute a genetic toolkit that is both deeply conserved within the plant lineage for some of them and also comprises a highly flexible gene set which has the potential to contribute to plant morphological innovations during evolution (Rast-Somssich et al., 2015).

3 MiRNA biogenesis and action provide entry points to regulate their activity

In order to understand how miRNAs regulate plant development, it's imperative to gain insight into their production, mechanisms of action, and how the modulation of these processes can influence miRNA activity. The biogenesis and action of miRNAs is a complex mechanism involving multiple actors. Some of these actors are specific to the miRNA pathway while others have roles in other biological processes. We will not detail here the growing knowledge of the mechanisms and actors at play, as very detailed reviews are available (Yu et al., 2017; Li and Yu, 2021). Briefly, miRNA genes are transcribed by the RNA polymerase II. Their transcripts are capped and polyadenylated and often spliced as they may extend over several exons. Part of the resulting pre-miRNA folds into a hairpin like structure that provides structural clues to direct a two-step maturation. First, the pre-miRNA transforms into the primary miRNA, characterized by a hairpin-like structure. Second, this primary miRNA undergoes further processing to form a small duplex structure, which consists of the miRNA itself and its complementary strand, often referred to as the miRNA* (miRNA star). This processing is mediated by the RNase DICER LIKE 1 and interacting proteins such as HYPONASTIC LEAVES 1 and SERRATE provide efficiency and precision to it. The miRNA is incorporated into the RNA INDUCED SILENCING COMPLEX (RISC) containing an ARGONAUTE (AGO) protein, often AGO1. Following the export to the cytoplasm, the miRNA containing RISC interacts with the target mRNA and triggers either its cleavage and/or inhibits its translation. MiRNA stability is also regulated: the miRNA is methylated at its 3' end to inhibit its degradation by preventing the addition of a short U tail, and nucleases may degrade the U-tailed or 3' non-methylated miRNA. It thus appears that the biogenesis of miRNAs and their activity is a very dynamic process with multiple levels of regulation (Meng et al., 2011). One central question that arises from this is to what extent

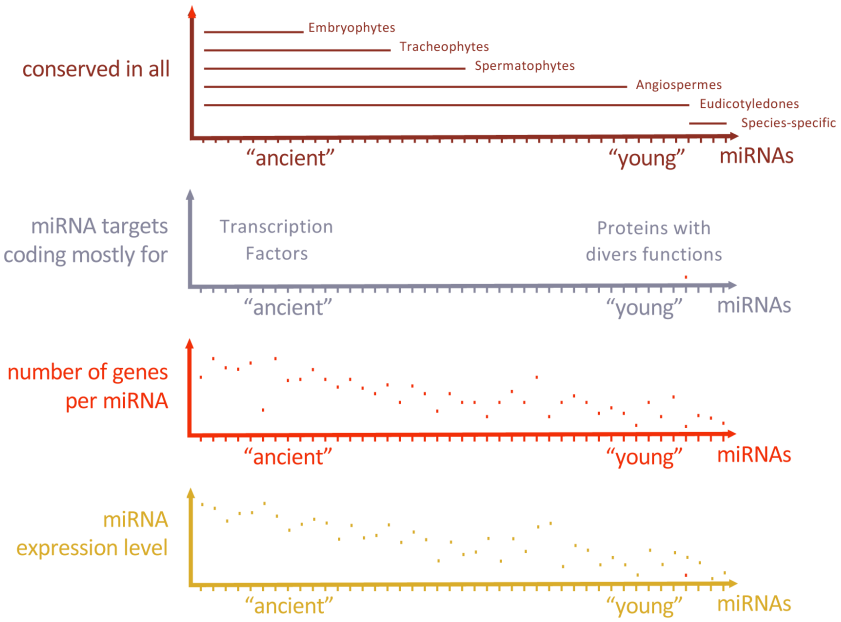

FIGURE 1
Characteristics of the typical miRNA repertoire of a dicotyledon. The miRNA repertoire is formed by ancient miRNAs shared with all Embryophytes and more recent ones shared with fewer lineages, some of them being specific to the species. Ancient miRNAs preferentially target genes coding for transcription factors while younger miRNAs target genes coding for proteins with much more diverse biological functions. The number of genes coding for ancient miRNA families and their expression levels tend to be higher than for younger miRNAs.

this general pathway can be specifically modulated for one or a subgroup of miRNAs. This is far from being understood although mutations in miRNA biogenesis actors may differentially affect the miRNA population. Consequently, the extent to which regulation of miRNA biogenesis and activity is utilized as a mechanism to govern plant development remains an unanswered question. In contrast, it is clear that transcriptional regulation of *MIRNA* gene expression is a major determinant of miRNA activity level. Indeed, expression of *MIRNA* genes can be regulated by specific transcription factors (Yang et al., 2021) and chromatin remodeling resulting from histone modifications or DNA methylation (Creasey et al., 2014; Xu et al., 2016; Hou et al., 2019). Such predominance of the transcriptional regulation level for *MIRNA* genes allows them to be fully integrated into gene regulatory networks (GRNs).

4 MiRNA integration in developmental gene regulatory networks

Plant development requires the integrated action of regulators forming GRNs. Pioneer work in *Escherichia coli* identified recurrent "network motifs" as the building blocks for larger networks (Milo et al., 2002; Shen-Orr et al., 2002). MiRNAs are embedded within the architecture of these GRNs as they target a diverse range of regulators including transcription factors (TF). In many cases, miRNA-mediated gene regulatory loops are formed that may be instrumental to network (Martinez et al., 2008). However, few studies have looked at miRNA function in plants from this network perspective. Here we will discuss a few examples that have been best characterized.

Plant architecture relies on the branching pattern which is dependent on the activity of axillary meristems. Zinc finger homeodomain TFs (ZF-HD TFs) with notably HB34 have been identified as regulators of plant architecture. Indeed, HB34 positively regulates *SPL10* while repressing *MIR157d* thus forming a feed-forward loop (FFL) (Lee et al., 2022). FFL are three-component patterns where a TF regulates the two other components that are already involved in regulatory interactions (Figure 2). The regulation of age-dependent cell death is another example for FFL including a miRNA (Kim et al., 2009). Here, *ETHYLENE INSENSITIVE 2* (*EIN2*) is required to induce *ORESARA1* (*ORE1*) expression which is targeted by miR164, and miR164 expression decreases with aging in an *EIN2*-dependent manner, forming a FFL. These two FFL examples represent coherent FFL where the sign of the indirect regulation path through the miRNA is the same as the sign of the direct regulation path (Figure 2). Work in *E. coli* suggests that coherent FFL generally introduce time-delay within networks. Therefore, positioning a miRNA as an intermediate component of coherent FFL in plants may contribute to network dynamics. Incoherent FFL are formed when the sign of the indirect regulation path through the miRNA and the sign of the direct regulation are different. Although less intuitive, incoherent FFL are also instrumental for developmental and environmental responses and may include a miRNA. For instance, nitrate response in the root triggers both miR393 and its target *AUXIN SIGNALING F-BOX 3* (*AFB3*) to activate auxin responses and promote lateral root development in an incoherent FFL (Vidal et al., 2010). This mechanism might provide a rapid developmental response at first, then a way of adjusting the level of response when optimal response has been reached, allowing it to adapt to environmental fluctuations.

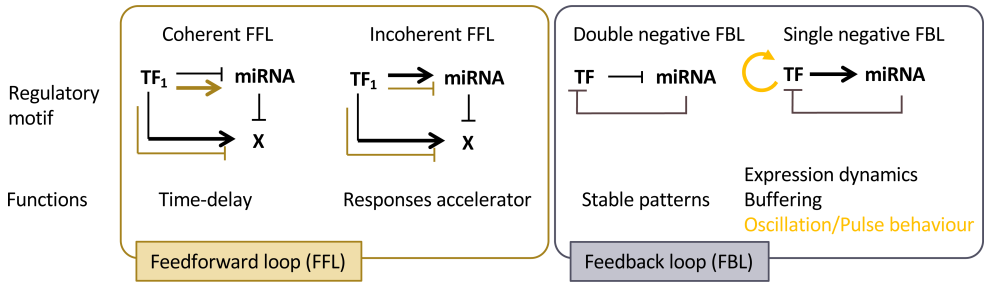

FIGURE 2
Regulatory motifs including miRNA are integrated into Gene Regulatory Networks. Feedforward (FBL) and Feedback loop (FBL) including a miRNA provide distinct functions which first depend on the sign of the regulatory interactions - coherent FFL when the TF regulates the gene X in the same way through both path; incoherent FFL when the TF regulates X in opposite way - then on the parameters of the regulatory motif such as expression levels and the stability of its components. For instance, oscillation or pulse behaviour may arise when an autoregulatory loop is present (orange arrow) depending on network parameters.

MiRNA are also integrated within networks through feedback loop motifs (FBL). These particular network motifs including miRNA are called composite FBL (Figure 2). As miRNAs exert negative regulatory effects on their targets, only two kinds of FBL exist: Single Negative (SN) - when the TF positively regulates a miRNA-, and Double Negative (DN) - when the TF negatively regulates the miRNA.

DN composite FBL results in complementary patterns that may allow spatialization of key developmental processes by maintaining terminal differentiated state (Johnston et al., 2005). In Arabidopsis flower development, specification between perianth and reproductive organs relies on such DN-FBL where the APETALA2 TF (AP2) activity is restricted to sepals and petals primordia by the action of miR172 in center of the floral meristem; LEUNING and SEUSS co-repressors restrict miR172 expression pattern in a AP2-dependent manner thus forming a composite FBL specifying flower whorls (Grigorova et al., 2011).

SN composite FBL may have various functions depending on the level of expression, the stability and/or additional regulatory interactions of its components. miRNA-TF feedback network motifs are over-represented in *C. elegans* and may confer robustness or oscillatory target gene expression patterns (Martinez et al., 2008). The nature of SN composite FBL implies that the TF activates the expression of the miRNA which is negatively regulating it in the same expression domain. If this regulation happens with a time delay, then the expression dynamic of the TF may be impacted. If TF autoregulatory activation occurs, then it can give rise to oscillatory or pulse behaviour depending on the parameters of the network motif. In some cases, the same cells express at the same time the TF and the miRNA. In this particular case, the function of the regulatory FBL could be to buffer TF expression to produce reproducible output. In plants, several TF and their inhibitory miRNA regulators are co-expressed in the same tissue such as miR160-ARF10/16/17, miR164-CUC1/2, miR156-SPL9/15 (Baker et al., 2005; Nikovics et al., 2006; Wang et al., 2008; Dai et al., 2021), but whether they are forming SN composite FBL and how these modules fine-tune gene expression remain to be determined.

5 MicroRNAs as mobile signals

Regulation of development involves coordination of basic cellular processes such as proliferation and differentiation at the organ or tissue levels. It also involves coordination across plant tissues or organs at the whole plant level. Communication is central for both processes and growing evidence enlightens how miRNAs may contribute to this. Indeed, like other small RNAs, miRNAs can be mobile at different distances: from cell-to-cell, between different plant organs or between individual (of the same species or not) through direct contact or through the environment (for recent reviews see eg Chen and Rechavi, 2022; Loreti and Perata, 2022; Yan and Ham, 2022). For instance, miR394 produced in the outer layer of the shoot apical meristem moves into deeper layers to define a region competent for stem cell formation (Knauer et al., 2013). Thus, miRNA mobility may provide positional information that contribute to patterning in plants. More precisely, the effects of such miRNA gradients can be at least twofold (Figure 3). During the establishment of adaxial/abaxial leaf polarity, miR166 is expressed within the abaxial epidermis. It then diffuses toward the uppermost cell layers, playing a role in creating a sharp boundary in the expression pattern of its targets, the Class III HD-ZIP genes, which are confined to the adaxial half of the leaf primordium. (Tatematsu et al., 2015; Skopelitis et al., 2017). In the developing root, a similar gradient of miR166 is formed as a result of its expression in the endodermis and movement to the root centre. However, here it leads to an opposite, increasing gradient of the Class III HD-ZIP expression levels from the endodermis to the more inner stellar tissues (Carlsbecker et al., 2010; Miyashima et al., 2011). Thus, mobile miR166 can have two different effects on its target expression pattern and thus differentiation: while in the leaf it leads to the sharp transition of the Class III HD-ZIP levels that contributes the formation of the contrasted abaxial and adaxial domains, in the root, it generates graded Class III HD-ZIP levels contributing to the differentiation of several cell types from endodermis, pericycle, proto- to metaxylem (Figure 3A). Such small range movement is a widespread mechanism affecting many miRNAs but which remains selective for the miRNAs moving, the

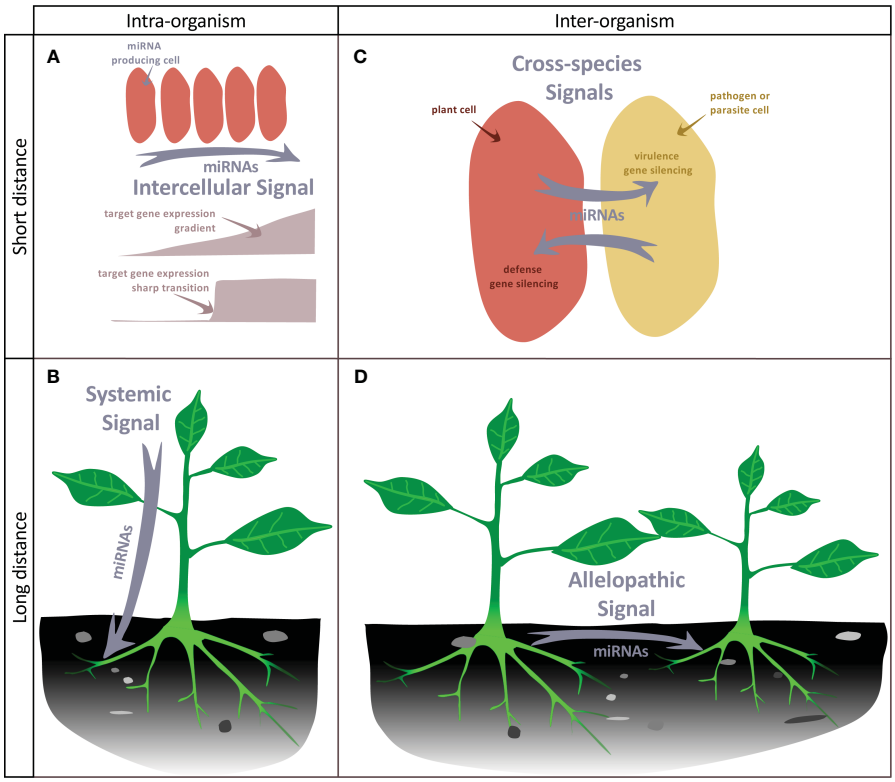

FIGURE 3
miRNAs as mobile signals. **(A)** miRNAs can move from cell-to-cell in an organ, generating a gradient of concentration that can either lead to an opposite gradient of expression of its target or to a sharp transition between cells expressing or not expressing the target. **(B)** miRNAs can move throughout the organism to generate a systemic signal. **(C)** During the interaction of a plant with a pathogen or a parasitic plant, miRNAs can be exchanged between the two neighboring cells to silence defense or virulence genes. **(D)** miRNAs can be released to the soil by plants and target genes of distant plants, thus possibly generating an allelopathic signal.

orientation of the movement and depends on the cell type (Skopelitis et al., 2018; Brosnan et al., 2019). This process is likely to occur through plasmodesmata, as it can be impeded by their closure via callose synthesis (Vatén et al., 2011). However, it's worth noting that other routes of diffusion may also be feasible. miRNAs can also move at a larger distance, between organs, possibly through the flux of phloem sieve. Such long-range miRNA movement constitute systemic signals that coordinate development and physiology at the whole plant scale (Figure 3B). For instance, miR172 and miR156 have been proposed to act as long-distance, graft- transmissible signals regulating tuberization in potato (Martin et al., 2009; Bhogale et al., 2014). Movement of miR399 from shoot to roots signals the onset of inorganic phosphate deficiency and represses its target PHO2 that regulates phosphate uptake from the root environment (Lin et al., 2008). Like for the small range movement, the favored form of transport is mature miRNAs, possibly free of bound AGO proteins (Brosnan et al., 2019; Dalmadi et al., 2019; Brioudes et al., 2021).

MiRNAs also constitute signals exchanged between two plants or with other organisms. For instance, miRNAs are transmitted from the parasitic plant *Cuscuta campestris* through specialized feeding structures called haustoria to the host plant. These parasitic miRNAs target conserved host genes that restrict parasite growth. Remarkably, parasitic miRNAs are typically unusually 22 nucleotides in length and have the capacity to initiate the production of secondary siRNAs, which can lead to the amplification of silencing mechanisms. (Shahid et al., 2018). Conversely, the host may also produce miRNAs to limit pathogens action: in response to *Verticillium dahliae* infection, cotton increases the expression and export of specific miRNAs to the pathogen to target virulence genes (Zhang et al., 2016). Whereas these examples illustrate miRNA exchanges between species in close physical connection, miRNAs can also be exchanged between distant individuals through a shared environment (Betti et al., 2021) (Figures 3C, D), with the potential to generate allelopathic signals. Again, response to these miRNAs requires the production of secondary siRNAs, which seems therefore a conserved mechanism to amplify weak signals provided by low amounts of mobile miRNAs. Cross-individual miRNA transport is thought to occur at least in part through extracellular vesicles containing different RNA binding proteins including AGO1, which may also contribute to the selective loading of a miRNA subpopulation (He et al., 2021).

6 Genetic strategies to approach miRNA functions during plant development and reproduction.

As discussed in the preceding sections miRNAs have multiple characteristics that give them the potential to play prominent roles during plant development and reproduction. In this context, the

royal road to decipher their roles is a functional approach analyzing the consequences of abnormal miRNA function through genetics. We will first critically assess the different strategies used to functionally analyze miRNAs before discussing their importance in developmental processes.

Probably the most widely used approach to establish functionally the roles of a miRNA, as it is amongst the easiest and fastest to perform, is to over express the miRNA of interest. However, it is important to note that this strategy, like any ectopic gain-of-function approach, might not provide a direct insight into the miRNA's normal physiological function. Indeed, using a promoter that differs from the endogenous promoter, either in terms of its expression pattern or level, can result in the down-regulation of targets that are distinct from the endogenous targets of this particular *MIRNA* gene, but are targets of other members of the same *MIRNA* gene family. Hence, miRNA overexpression is rather a way to reveal the collective roles of the miRNA targets which are down-regulated by miRNA over expression. Yet, such an approach remains indicative of the possible roles of the miRNA and is at least a very good way to experimentally confirm target gene predicted *in silico*, which can however be blurred by feedback mechanisms (Schwab et al., 2005).

Another widely used strategy is to express a target gene that is made resistant to the miRNA by introducing silent mutations in the miRNA binding site either via transgenesis or genome-editing (Lin et al., 2021b). While this strategy is highly effective in uncovering the impact of a miRNA on the expression and function of a specific target, it does not encompass its effect on multiple targets, thus overlooking the full spectrum of the miRNA's functions. Nevertheless, this strategy is still the most effective for bridging the gap between a miRNA and a phenotype through its effects on one or several particular targets.

Another widely-used strategy based on transgenics to get access to the function of a miRNA is to express a decoy target to partially inactivate the miRNA. These decoy targets are RNA molecules that contain one or several stretches highly complementary to the miRNA that, however, have at the cleavage site a mismatch loop preventing cleavage while still allowing the binding of the miRNA. Therefore, they compete for the binding of miRNAs to endogenous targets, sequester them and lead to their degradation. A first generation of such decoy mRNAs called MIMICs was based on the long non-protein coding RNA IPS1 (INDUCED BY PHOSPHATE STARVATION 1) and contains one miRNA binding site (Franco-Zorrilla et al., 2007; Todesco et al., 2010), while a second generation called Short Tandem Target MIMICs (STTMs) contains two binding sites separated by a spacer (Yan et al., 2012). Other artificially-engineered RNAs (miRNA sponges) or endogenous RNAs (circular RNAs or long non coding RNAs) may also sequester miRNAs through their binding sites (Reichel et al., 2015; Tang et al., 2021; Zhou et al., 2021; Liu et al., 2023). These approaches are easy to set up when stable plant transformation is feasible, while transient transformation using a virus-based delivery system can be used too (Yan et al., 2014). Collections of MIMICS and STTMs lines are available for Arabidopsis, tomato, rice and maize (Todesco et al., 2010; Peng et al., 2018). Such approaches go with the advantages of generating pseudo-allelic series in which different levels of miRNA inactivation occur, are transferable to different species in the case of conserved miRNAs and allow targeting in a dominant fashion multiple members of a *MIRNA* gene family. The reverse of the coin is that the contribution of individual gene members of the *MIRNA* family cannot be investigated. Also, the efficiency of such strategies depends on the approach used and most importantly on the targeted miRNA, and remains unpredictable (Reichel et al., 2015). In addition, miRNA down-regulation is only partial. For instance, a 65% reduction of miR164 was observed in STTM tomato seedlings, while a 90% reduction was observed in the pericarp of developing tomato fruits when only *MIR164a*, one of the 4 *MIR164* genes was inactivated (Gupta et al., 2021). In addition to being incomplete, the level of miRNA inactivation may vary within a plant both temporally or spatially (for instance due to variation of the activity of the promoter driving the MIMIC expression). Hence, while MIMIC and STTM approaches are highly valuable for assessing the general functions of a miRNA in plant development, they do exhibit limitations when it comes to elucidating the specific roles of individual *MIRNA* genes or uncovering their complete range of functions.

All the methods discussed above have been developed to overcome the initial difficulty to identify mutant lines impaired in miRNA production. Such a difficulty results first from the fact that *MIRNA* genes form families, sometimes with numerous members and that their roles are often at least partially redundant. Therefore, mutants for each member have to be identified and higher order mutants may have to be generated. In addition, finding miRNA insertion (T-DNA or transposon) mutants by chance can be unsuccessful as mature miRNA and their pri-miRNA precursors are small. Alternatively, point mutations generated by chemical mutagenesis need to affect the mature miRNA as point mutations elsewhere in the pri-miRNA do not necessarily impact its maturation into miRNA (Mateos et al., 2010). Nevertheless, mutants in *MIRNA* genes were identified shortly after the identification of plant miRNAs as the genetic basis of developmental mutants previously characterized, thus providing the first indications that miRNAs contribute to developmental processes (Baker et al., 2005; Chuck et al., 2007; Liu et al., 2010). However, with the exception of the *MIR164* and *MIR159* families in Arabidopsis (Sieber et al., 2007; Allen et al., 2010), no comprehensive analysis of all members of a *MIRNA* gene family could be performed due to the lack of mutants. The reports that *MIRNA* gene mutation could be triggered by CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins)- based genome editing technologies (Jacobs et al., 2015; Zhao et al., 2016) was a game changer in this field and was rapidly applied to study the developmental roles of *MIRNA* genes (Table 1) (for a more comprehensive review about CRISPR applications on *MIRNA* genes see Deng et al., 2022). In a few years, genome editing of *MIRNAs* has come to a maturity. Methods to design the strategy and identify mutants have been improved. For instance, ways to test the efficiency of guide RNAs in protoplasts before stable transformation have been developed (Zhou et al., 2017). Double targeting to induce large deletions at the *MIRNA* locus is often used as single indels resulting from single targeting do not always affect miRNA functions (Zhou et al., 2017;

TABLE 1 MIRNA gene mutants and their phenotypes.

miRNA	Species	MIRNA number (a)	MIRNA	Mutant (if forward genetics)	Mutant origin	Target(s)	Phenotype of MIRNA mutant	References
miR156	Arabidopsis	10	*MIR156a, c*		T-DNA	*SPL3, SPL9, SPL13*	earlier phase transition	Yang et al., 2013
	Rice	12	*MIR156d, e,f,g,h,i*		CRISPR	*SPL*	reduced branching	Miao et al., 2019
			MIR156a, b,c,k,l				increased seed dormancy and longetivity	
miR159	Arabidopsis	3	*MIR159a, b*		T-DNA	*MYB33, MYB36*	reduced apical dominance, curled leaves, smaller siliques and seeds	Allen et al., 2007
	Tomato	6*	*MIR159a*		CRISPR	*SlGAMYB2*	larger fruit	Zhao et al., 2022
miR160	Arabidopsis	3	*MIR160a*	*floral organs in carpels (foc)*	DS transposon	*ARF10, ARF16, ARF17*	increased leaf serration, aborted seeds, abnormal siliques	Liu et al., 2010
			MIR160a		CRISPR	*ARF10, ARF16, ARF17*	increased leaf serration, curled petals, aborted seeds	Bi et al., 2020; Ouyang et al., 2020
	Tomato	4*	*MIR160a*		CRISPR	*ARF10, 16, 17*	wiry leaves, misshapen and sterile flowers	Damodharan et al., 2018
			MIR160b		CRISPR	*ARF10, 16, 17*	normal	Damodharan et al., 2018
	Marchantia polymorpha		*MIR160*		CRISPR	*ARF3*	proliferative outgrowths, fewer gemma cups and air pores	Flores-Sandoval et al., 2018
miR164	Arabidopsis	3	*MIR164a*		T-DNA	*CUC2*	increased leaf serration	Nikovics et al., 2006
			MIR164b		T-DNA	*CUC1/CUC2*	normal	Mallory et al., 2004
			MIR164c	*early extra petals1 (eep1)*	Tag1 transposon	*CUC1/CUC2*	extra petals, pistil defects	Baker et al., 2005
	Strawberrry	3	*MIR164a*		EMS	*CUC2a*	increased leaf and petal serration, deformed carpels	Zheng et al., 2019
	Tomato	4*	*MIR164a*		CRISPR	*NAM2, NAM3*	accelerated fruit ripening, smaller fruits	Lin et al., 2022; Gupta et al., 2021
			MIR164b		CRISPR	*GOB*	extra cotyledons, smaller and simplified leaves, bushy, sterile	Gupta et al., 2021
			MIR164d		CRISPR		normal	Gupta et al., 2021
miR167	Arabidopsis	4	*MIR167a*		CRISPR		late flowering, sterile due to defects in anther dehiscence and ovule development	Yao et al., 2019
			MIR167b, c,d		CRISPR	*ARF6, ARF8.*	normal	Yao et al., 2019
miR168	Rice	2	*MIR168a*		CRISPR	*AGO1a,b,d AGO18*	bushy plants, small spiklets and seeds	Zhou et al., 2022
miR169	*Antirrhinum majus*		*MIR169*	*fistulata*	MITE transposon	*NF-YA*	stamenoid petals	Cartolano et al., 2007
			MIR169	*blind*		*NF-YA*	stamenoid petals	

(Continued)

TABLE 1 Continued

miRNA	Species	MIRNA number (a)	MIRNA	Mutant (if forward genetics)	Mutant origin	Target(s)	Phenotype of MIRNA mutant	References
	Petunia hybrida				dTPH1 transposon			Cartolano et al., 2007
miR171	Rice	9	*MIR171f*		CRISPR	*SCL6-I, SCL6-II*	lower total grain weight and filling rate	Um et al., 2022
miR172	Arabidopsis	5	*MIR172a*		T-DNA		delayed adaxial trichomes	Wu et al., 2009
			MIR172d	not named	EMS		enhances *agamous-10*, short gynoceum with sometimes 3 carpels	Yumul et al., 2013
			MIR172a-e		CRISPR		delayed adaxial trichomes, larger SAM, short internodes, more branches, floral meristem termination defects, serrated cauline leaves.	Ó'Maoiléidigh et al., 2021; Lian et al., 2021
	Maize	5	*MIR172e*	*tasselseed4*	Helitron transposon	*AP2*	irregular branching of inflorescence, feminized florets of the tassel	Chuck et al., 2007
	Tomato	8*	*MIR172c*		CRISPR	*AP2-like*	greening of sepals and petals	Lin et al., 2021a
			MIR172d		CRISPR	*AP2-like*	extra floral organs in whorl 2 and 3, petal and stamen conversion into sepaloids	Lin et al., 2021a
miR319	Arabidopsis	3	*MIR319a*		EMS	*TCP2, 3, 4, 10, 24*	narrow and short petals, defective anther development	Nag et al., 2009
			MIR319b		T-DNA	*TCP2, 3, 4, 10, 24*	reduced leaf serration, phenotype more severe when combined with mir319a	Koyama et al., 2007
miR390	Rice	1	*MIR390*		TALEN	*TAS3*	no embryonic shoot apical meristem or meristem maintenance defects.	Bi et al., 2020
miR394	Arabidopsis	2	*MIR394b*	not named	EMS	*LCR*	shoot apical meristem defects when combined with *ago10* mutation	Knauer et al., 2013
miR396	Arabidopsis	2	*MIR396a MIR396b*		CRISPR	*GRF1,2,3,4,7,8,9, CIB4*	larger leaves, earlier phase transition	Hou et al., 2019
	Rice	8	*MIR396e, f*		CRISPR	*GRF1-10, 12*	shorter internodes, larger leaves and grain	Miao et al., 2020
miR408	Rice	1	*MIR408*		CRISPR	*UCL8*	smaller grain	Yang et al., 2021
miR482	Coton	36**	*MIR482a-h,k,l*		CRISPR	*NLR*	lower disease index in response to *Verticillium dahliae* infection	Zhu et al., 2022
miR529	*Marchantia polymorpha*		*MIR529c*		CRISPR	*SPL2*	spontaneous transition to the reproductive phase	Tsuzuki et al., 2019
miR845	*Arabidopsis*	2	*MIR845a*		T-DNA			Borges et al., 2018
miR2118	*Rice*	1	*MIR2118*		CRISPR	*PHAS lincRNA*	male and female sterility, anther wall defects	Araki et al., 2020
miFRH1	*Marchantia polymorpha*		*MIFRH1*		CRISPR	*RSL1*	increased rhizoid clusters	Thamm et al., 2020

(a) MIRNA gene numbers are from miRBase (release 22.1, Kozomara et al., 2019), from Arazi and Khedia, 2022 (*) or Zhu et al., 2022 (**).

Lukan et al., 2022). Nevertheless, quantifying precisely the effects of the induced mutations on miRNA function (a combined effect of *MIRNA* expression level, maturation, stability, target recognition and inhibition) remains a very challenging task. One pragmatic way to test it is to compare the effects of wild-type and mutant *MIRNA* gene overexpression (eg Ó'Maoiléidigh et al., 2021; Lian et al., 2021). Such *MIRNA* mutation via genome editing can be used for model species and crops including polyploids (Lukan et al., 2022;

Zhu et al., 2022). This system provides a high versatility: depending on the specificities of the used guides, it can either target a single *MIRNA* gene of a multigene family to provide the opportunity to generate individual mutants for each member of a *MIRNA* family and combine them progressively via crossing (for instance Lian et al., 2021 constructed 24 combinations for 5 different *MIR172* genes) or target multiple genes simultaneously using multiple specific guides (Zhu et al., 2022).

7 From specialization to full genetic redundancy of *MIRNA* genes

The identification of *MIRNA* genes as the genetic basis of mutants initially identified for their developmental defects unambiguously showed that some individual *MIRNA* genes have specific roles that are not masked by genetic redundancy. For instance, the *early extra petal* mutation leading to more petals was shown to result from the reduced expression of *MIR164c*, one of the three *MIR164* Arabidopsis genes (Baker et al., 2005). Such a link between *MIRNA* genes and developmental phenotypes was not limited to the model species Arabidopsis as in maize *tasselseed4* mutants have irregular branching in the inflorescence and feminization of the tassel due to mutations in a *MIR172* gene (Chuck et al., 2007), while a mutation of a *MIR169* gene leads to petal conversion into staminoid structures in both *Antirrhinum majus* and *Petunia hybrida* (Cartolano et al., 2007). However, a more systematic analysis of the roles and interactions between different *MIRNA* gene members remains limited to species with dense T-DNA or transposon mutant collections such as Arabidopsis or for which genome editing was well-developed (mostly rice and tomato). The discovery of mutants in multiple *MIRNA* genes within the same family has revealed that they can have roles that range from specific involvement in certain organs or developmental processes to exhibiting quantitative additive effects or even complete genetic redundancy. We will illustrate these findings below.

In line with the forward genetic approach described above, reverse genetics also identified individual *MIRNA* genes with important roles in specific developmental contexts. As an illustration, a strong specialization was shown for *MIR164* genes in Arabidopsis and tomato. In parallel to *MIR164c* that determines Arabidopsis petal number via the regulation of the *CUC1* target (Baker et al., 2005), *MIR164a* regulates leaf serration via its effect on the *CUC2* target (Nikovics et al., 2006). This also suggests that specialization of some *MIRNA* gene members could go along with a preferential regulation of some targets in a specific developmental context. In tomato, *MIR164b* is the main regulator of meristem-to-organ and organ-to-organ boundary specification, stem internode elongation, and flower abscission zone development, while *MIR164a* is required for division and maturation in tomato fruits (Gupta et al., 2021). Such specialization of the *MIRNA* genes can be due to their specific expression patterns: *MIR164a* is specifically expressed in the leaves in domains overlapping with *CUC2* (Nikovics et al., 2006) while *MIR164c* is the only *MIR164* gene to be expressed in the floral meristem where the petals form (Sieber et al., 2007). In addition to differences in gene expression levels or patterns, differences in miRNA maturation can also contribute to variations in the importance of individual *MIRNA* genes of the same family, as illustrated by Arabidopsis *MIR167*. Within the four *MIR167* members, *MIR167a* has the major role as its mutation leads to a strong phenotype including anther dehiscence and ovule development defects (Yao et al., 2019). In contrast, the *mir167bcd* triple mutant is very similar to wild type. Such differences in the developmental contribution of the *MIR167* members may be due to a less efficient maturation of the *MIR167b,c,d* precursors compared to *MIR167a* as only *MIR167a* has strong effects when ectopically expressed while *MIR167b,c,d* have no or weak effects (Wu et al., 2006).

The combination of multiple mutations has shown that many *MIRNA* genes have redundant roles, with sometimes quantitative effects. Even in the case of a strong specialization, a certain level of genetic redundancy is observed. In tomato, *MIR164a* and *MIR164b* that have specific functions have also redundant roles as the double *mir164ab* mutant has very strong seedling defects not observed in any of the single mutants (Gupta et al., 2021). At the more extreme case, full functional redundancy between *MIR159a* and *MIR159b* results from similar expression patterns of these two genes and a similar mature miRNA sequence. As a result, each of the single mutant shows a wild-type phenotype while the *mir159ab* double mutant has hyponastic leaves, stunted growth, reduced apical dominance, reduced fertility and seed set (Allen et al., 2007). More generally, redundancy has been shown between different members of *MIR156*, *MIR164*, *MIR167*, *MIR172*, *MIR396* in Arabidopsis (Sieber et al., 2007; Yang et al., 2013; Hou et al., 2019; Ó'Maoiléidigh et al., 2021; Lian et al., 2021), *MIR172* in tomato (Lin et al., 2021a) and seems to be the general rule, at least to a certain level. In some instances, it has been reported that *MIRNA* genes control a specific character in a quantitative way. An emblematic illustration is shown by Ó'Maoiléidigh et al. (2021) who observed that the number of Arabidopsis leaves produced before flowering gradually increases when mutations of the five *MIR172* genes are stacked one by one from the single *mir172a* mutant to the quintuple mutant. As for *MIR159a* and *MIR159b* genes, the basis of this redundancy is an overlap of *MIRNA* gene expression and similar effects of the miRNAs produced. An unexplored question related to *MIRNA* gene redundancy is the fine role of the different miRNA isoforms. Based on predictions, their efficiency towards their targets may be different and in the most extreme case, they may have different targets.

In the following sections, our objective is to discuss the roles of miRNAs during plant development and reproduction. Rather than aiming to provide an exhaustive list of roles, we will critically assess their significance in four key processes. This assessment will be primarily based on the analysis of *MIRNA* mutants, but we will also explore their connections with other regulators and their effects on target genes.

8 miRNAs, quantitative regulators of plant growth

Growth, when it operates at a global scale, acts as the driving force behind plants continual development. However, when growth is differential and precisely regulated at a local level, it becomes the

mechanism through which morphogenesis, the development of new forms, takes place. From a more mechanistic point of view it combines different cellular processes such as cell proliferation and cell expansion that are regulated by multiple plant hormones. MiRNAs contribute to the regulation of growth at these two scales.

In Arabidopsis, miR159 targets seven genes encoding MYB transcription factors that contribute to the regulation of the expression of genes in response to gibberellins (GA). MiR159 acts by clearing the expression of two of them, *MYB33* and *MYB65*, from vegetative tissues and limits their expression to anthers and seeds. Ectopic expression of *MYB33* and *MYB65* in vegetative tissues of the *mir159ab* double mutant leads to a reduced growth due to a reduced cell proliferation which is not totally compensated by an increased cell expansion (Allen et al., 2007; Alonso-Peral et al., 2010). Therefore, miR159 appears to act as a molecular switch in Arabidopsis to shut-off its targets in inappropriate tissues. In tomato, miR159 has a role during fruit growth as a mutation of *MIR159a* leads to larger, less elongated fruits. Here miR159, by targeting the *SlGAMYB2* gene coding a transcription factor that represses the GA biosynthetic gene *SlGA3ox2* indirectly promotes GA biosynthesis (Zhao et al., 2022). Because miR159 expression is induced by GAs (Achard et al., 2004), this forms a positive feedforward loop that may contribute to fruit size regulation. Thus, miR159 acts as a modulator of growth, either at the whole plant level as in Arabidopsis or the organ level like in tomato.

In Arabidopsis, differential growth at the leaf margin leads to the formation of small serrations. The balance between co-expressed *MIR164a* and *CUC2* in the sinus of the serration determines the size of the serration, possibly by controlling their growth speed (Nikovics et al., 2006; Kawamura et al., 2010; Bilsborough et al., 2011; Maugarny-Calès et al., 2019). As a consequence, if *miR164* function is compromised, Arabidopsis leaves form overdeveloped serrations, a phenotype that is also observed in strawberry (Zheng et al., 2019). Thus, by fine tuning growth, miRNAs control overall plant and organ size and also contribute to the fine regulation of plant organ shape.

9 miRNAs, regulators of plant organ patterning and identity

Morphogenesis involves the formation of new organs according to a precise spatial and temporal pattern, while also ensuring that they acquire their correct identity. As discussed above, *MIR164c* is required to regulate petal number in Arabidopsis (Baker et al., 2005), while in tomato *MIR172d* is limiting petal and stamen numbers (Lin et al., 2021a). MiRNAs have also been shown to contribute to patterning at the cellular scale as shown for the rhizoids in the liverwort *Marchantia polymorpha*. Rhizoids are single cell outgrowth that develop from a layer of epidermal cells and tend to be spaced and form only unfrequently linear clusters. Rhizoid formation is promoted by the basic loop helix loop transcription factor ROOT HAIR DEFECTIVE SIX-LIKE1 (RSL1) which activates the expression of its negative regulator the miRNA FEW RHIZOIDS1 (FRH1). This negative feedback loop contributes to patterning the rhizoid by generating a lateral inhibition mechanism (Thamm et al., 2020). Whether the role of FRH1 in mediating lateral inhibition involves diffusion of the miRNA it-self from the rhizoid precursor cell where it is expressed or diffusion of a downstream signal is not known yet.

Several examples also illustrate the roles of miRNAs in the regulation of organ identity. For instance, in *Antirrhinum majus* and *Petunia hybrida* mutant for *MIR169*, the acquisition of petal identity is compromised, resulting in an increase in stamen identity. This is due to elevated and slight expansion of the C-function expression (Cartolano et al., 2007). In tomato, petals and stamens are converted into sepaloid organs when *MIR172d* is reduced, suggesting that the AP2 genes that are consequently upregulated may both repress C-function genes to the most inner part of the floral meristem and repress B-function genes (Lin et al., 2021a). These two examples indicate that *MIRNAs* may contribute to provide positional information necessary for the correct acquisition of organ identity during flower development. A more extreme case of organ identity is shown by the role of *MIR172e*/*Tasselseed4* (*ts4*) in maize (Chuck et al., 2007). In *ts4* mutants, the typically male flowers in the tassel fail to undergo pistil abortion, resulting in feminization and seed formation. A similar phenotype results from dominant *ts6* mutations. *Ts4* encodes a member of the *MIR172 f*amily, while the *ts6* mutation lies in the miR172-binding site of an *AP2* gene. Compromised miR172 action does not have any discernible effect on the mRNA level or cleavage of its target but leads to the ectopic presence of the target protein in the pistil that fails to abort. Therefore, miR172 limits AP2/TS6 presence in male flowers to abort pistil development and acts mostly by repressing its translation, a mechanism also occurring in Arabidopsis (Aukerman and Sakai, 2003; Chen, 2004).

10 miRNAs, regulators of plant life cycle

Phase transition from juvenile to adult is important for the plant life cycle as it allows the plant to acquire its reproductive potential. MiR156 plays pivotal roles during this transition, maintaining the juvenile phase by repressing specific *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) activities. Indeed, SPL transcription factors have specific roles during vegetative development with SPL9 promoting adult state (Schwarz et al., 2008). Another miRNA, miR172 is acting downstream of miR156 to promote adult state (Wu et al., 2009) by repressing the activity of APETALA2-like transcription factors such as TARGET OF EAT 1 (TOE1) and TOE2 (Aukerman and Sakai, 2003; Jung et al., 2007; Wu et al., 2009). SPL9 is likely to induce *MIR172b* and *MIR172c* but not *MIR172a* to coordinate juvenile to adult transition (Wu et al., 2009; Lian et al., 2021). Thus, sequentially operating MIRNAs ensure the juvenile to adult transition, miR156 and miR172 having opposite effects on phase transition.

Floral transition is crucial for plant reproductive success; hence it needs to be tightly controlled to ensure flowering at the right time. Once again miR172 plays an important role here. Specific combinations of *MIR172* genes responding to different endogenous

or exogenous signals (such age, temperature, photoperiod) promote the transition to flowering (Lian et al., 2021). For instance, low ambient temperature modulates flowering by controlling the expression of *MIR172c* and *MIR172d* at both transcriptional and post-transcriptional levels (Jung et al., 2007). Additionally, specific *SPL* genes are acting as intermediate between the environmental or endogenous factor and *MIR172* gene activation, therefore forming a complex network of partially interdependent regulation (Lian et al., 2021). As individual *MIR172* genes integrate multiple signals, the contribution of MIR156-dependent SPL regulation remains to be determined.

MiR172 is at the core of phase transition in plants suggesting that it is required for plant growth and development. The analysis of *mir172* multiple CRISPR mutants tells a different story as *mir172* quintuple mutant is viable and can even produce large amounts of seeds when grown outdoors (Lian et al., 2021). This is showing that, although miR172 is required for SAM formation and control meristem size through a AP2/ARF3 module (Jung et al., 2007; Ó'Maoiléidigh et al., 2021), it is not absolutely required for seed-to-seed life cycle in Arabidopsis and that additional miR172-independent pathways exist.

11 miRNAs, essential factors for plant reproduction

To fulfill their developmental cycle, plants need to sustain continuous growth by maintaining an active pool of stem cells in meristems and develop male and female reproductive structures. miRNAs are essential for all these events. In rice, mutants for the unique *MIR390* gene show a shootless phenotype and could therefore not be maintained as homozygous mutants (Bi et al., 2020). Mutation of *MIR394* in Arabidopsis severely enhances the shoot apical meristem defects of *ago10-1* (Knauer et al., 2013). Two families of miRNAs, miR160 and miR167 that modulate auxin responses via the targeting of different ARFs are required for proper ovule, stamen or seed development in Arabidopsis and tomato (Liu et al., 2010; Damodharan et al., 2018; Yao et al., 2019; Bi et al., 2020). For these two families, the disruption of a single member is sufficient to induce a strong reproductive defect. In addition, mutations of other miRNA genes such as *MIR164b* in tomato, *MIR159a* or *b*, *MIR319a* in Arabidopsis and *MIR2118* in rice reduce fertility (Allen et al., 2007; Nag et al., 2009; Araki et al., 2020; Gupta et al., 2021).

Beside controlling morphogenesis, miRNAs also have an important contribution to genome homeostasis. Multiple miRNAs, including conserved miRNAs that target developmental genes such as miR159 or miR172, also target different transposons and trigger the production of epigenetically activated small interfering RNAs (easiRNAs), which also target transposons. This pathway is particularly active in mutants such as decreased *dna methylation 1* (*ddm1*). Here, transposons are transcriptionally derepressed and the easiRNA pathway is required for normal plant fertility (Creasey et al., 2014). The production of easiRNAs is also triggered in a dose dependent manner by *MIR845b* which is specifically expressed in pollen. More precisely, easiRNAs produced in the vegetative nucleus move to the neighboring germ cell (Borges et al., 2018). A T-DNA insertion in *MIR845b* reduces miR845 and easiRNA accumulation and leads to a reduction of the triploid block which drives a reproductive barrier between species with different chromosome numbers. Therefore, the contribution of miRNAs contribution could extend from their role in the reproduction of individual plants to a broader role in plant evolution and speciation.

12 The role of microRNAs during plant versus animal development.

Similar to plants, animal miRNAs are organized into families, with some families being highly conserved while having at the same time a high dynamic in the birth, evolution and death of new genes (Niwa and Slack, 2007; França et al., 2016). In contrast to plant miRNAs that mostly bind to unique sequences with a high complementarity to produce strong impact on gene expression, animal miRNAs often have multiple binding sites and their action on gene expression is more limited. In fact, the action of animal miRNAs is cooperative, meaning that multiple binding sites are required to have a strong effect on gene expression. Furthermore, more limited complementarity is sufficient between an animal miRNA and its target, which has the important consequence of increasing the number of genes that are targeted by each miRNA. Therefore, essential differences exist between plant and animal miRNAs on their downstream gene target network and their quantitative effects on target expression. Functional analysis on animal miRNA gene families have led to the idea that only a few of them are essential for development (Alvarez-Saavedra and Horvitz, 2010), a view that however could be revisited with more thorough genetic analysis. Systematic analysis of animal miRNAs and their target expression studies have led to the proposal of a bimodal role of miRNAs during development (Alberti and Cochella, 2017; Avital et al., 2018). On one hand, miRNAs expressed during early embryogenesis would preferentially have a strong impact on their target expression, leading often to complementary patterns between the miRNA and its targets. These miRNAs often regulate basic mechanisms such as proliferation, apoptosis or cell signaling. On the other hand, another group of is expressed at later stages, during the process of differentiation, and exhibits cell-type-specific patterns of expression. These miRNAs have a more limited effect on the expression of their targets and may contribute to robustness of their expression (Ebert and Sharp, 2012; Cassidy et al., 2013). As discussed above, plant miRNAs may have similar effects on their target genes, being essential contributors of their expression pattern or fine regulators of their levels. Therefore, plant and animal miRNAs are likely integrated in regulatory networks of similar architectures that allow them to have similar molecular outcome despite differences in their mode of action or the number of targets. However, because plant development is a continuous process that result in an organism having at one moment organs at different developmental stages, the separation between miRNAs regulating early patterning and growth and those involved in later differentiation can not be done on a temporal basis as in animals but rather on a spatial basis. Therefore, the apparent differences in

miRNA roles observed during plant and animal development are more likely to result from the distinct logic of development that exists in these organisms rather than reflecting true fundamental differences in miRNA function.

13 Conclusion

Our current knowledge unequivocally indicates that numerous miRNAs are a pillar of plant development and reproduction. Their inactivation leads to plants that are unable to carry out critical steps such as meristem maintenance, production of reproductive organs or genome homeostasis. Notably, the requirement for miRNAs in plant development is not strictly linked to gene family size, as some single genes are essential, while certain larger families appear to have milder contributions. Despite important progress in the last years, our understanding of miRNAs' roles remains incomplete. Within a *MIRNA* gene family, functional data are often missing for one or several members, and some entire families are yet to be fully explored. Furthermore, such functional analysis tends to concentrate on conserved miRNAs, neglecting for the moment more recent miRNAs that could have functions more limited to some developmental particularities in a few species. In this context, genome editing has proven to be an indispensable tool to go deeper into functional analysis and gain further insights into the functions of miRNAs in plant development and reproduction.

Beside such broad-scale functional analysis, a more comprehensive understanding of the roles of miRNAs requires a deeper comprehension of their position and effects on GRN. This is a question shared with other genetic regulators and includes the key issues of the regulation of their expression. However, due to the unique characteristics of miRNAs, such as their biogenesis and mode of action, it also raises specific questions that are exclusive to these small RNA molecules, such as their quantitative effect on target expression and more generally on GRN dynamics.

Author contributions

PL: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. NA: Conceptualization, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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