Review

New Phytologist

Tansley insight

Mobile plant microRNAs allow communication within and between organisms

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Received: *22 April 2022*
Accepted: *27 June 2022*

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Contents

Summary 2176

I. MicroRNAs: molecules regulating posttranscriptional gene silencing 2176

II. MicroRNAs can act as mobile molecules 2177

III. MicroRNA mobility in plant–pathogen interactions 2177

IV. MicroRNAs as plant-to-plant signaling molecules 2178

V. Conclusions 2180

Acknowledgements 2181

References 2181

New Phytologist (2022) **235:** 2176–2182
doi: 10.1111/nph.18360

Key words: Arabidopsis, cross-kingdom RNAi, exogenous RNA, miRNA, RNA interference, small RNA.

Summary

Plant microRNAs (miRNAs) are small regulatory RNAs that are encoded by endogenous miRNA genes and regulate gene expression through gene silencing, by inducing degradation of their target messenger RNA or by inhibiting its translation. Some miRNAs are mobile molecules inside the plant, and increasing experimental evidence has demonstrated that miRNAs represent molecules that are exchanged between plants, their pathogens, and parasitic plants. It has also been shown that miRNAs are secreted into the external growing medium and that these miRNAs regulate gene expression and the phenotype of nearby receiving plants, thus defining a new concept in plant communication. However, the mechanism of miRNA secretion and uptake by plant cells still needs to be elucidated.

I. MicroRNAs: molecules regulating posttranscriptional gene silencing

MicroRNAs (miRNAs) are small, 20–22 nt long noncoding RNAs that regulate gene expression in most eukaryotes by posttranscriptional gene silencing (PTGS; Bologna & Voinnet, 2014). miRNAs are only one of many classes of small RNAs that act as a guide for silencing gene expression, both at a transcriptional and posttranscriptional level (Singh *et al.*, 2018). RNA interference (RNAi) modulates gene expression by either inducing messenger RNA (mRNA) degradation or by inhibiting its translation. mRNA target slicing requires a high degree of sequence complementarity between the miRNA and the target sequence (Rogers & Chen, 2013), whereas translation inhibition can still take place with a lower degree of complementarity (Li *et al.*, 2013). However, certain plant miRNAs exhibiting perfect or near-perfect complementarity to a single target site can repress mRNA expression predominantly at the translation level (Brodersen *et al.*, 2008). The extensive sequence complementary between plant miRNAs and their targets results in target mRNA degradation as the preferential RNAi pathway occurring in plants (Bologna & Voinnet, 2014). miRNAs play a crucial role in plant developmental processes, such as

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patterning of the embryo, meristem, leaf, and flower (D'Ario *et al.*, 2017), as well as the plant's response to biotic and abiotic stresses (Khraiwesh *et al.*, 2012). Another class of small RNAs (sRNAs) that play an important role in RNAi in plants are secondary small interfering RNAs (siRNAs). Secondary siRNAs are produced from a double-stranded RNA (dsRNA) precursor, whose synthesis is triggered by an upstream miRNA or sRNA-dependent mRNA cleavage event followed by the production of a duplex RNA through RNA-dependent RNA polymerase (RDR) activity, known also as RDR6-dependent transitivity (Carbonell, 2019). Interestingly, miRNA can lead to the production of secondary siRNAs from its mRNA target, thus amplifying the silencing of the target itself (de Felippes, 2019).

II. MicroRNAs can act as mobile molecules

The effects of miRNAs can be cell autonomous or cell nonautonomous (Voinnet, 2022). The latter occurs when the effect of the miRNAs extends beyond the cell that has produced it. Evidence for a cell-nonautonomous silencing signal was observed in several of the key studies that led to the discovery of RNA silencing (Pyott & Molnar, 2015), thus indicating miRNA mobility in the plant. Not all RNAs have the same ability to move from their site of synthesis and act elsewhere. RNA mobility is often directional and/or restricted to some tissues, and some sRNAs can move over longer distances (Voinnet, 2022). Given that ARGONAUTE1 (AGO1) is not a mobile molecule, movement of siRNAs or miRNAs in the plant occurs as AGO-free RNA molecules (Devers *et al.*, 2020). However, AGO proteins abundance is important to define the range of mobility of a given siRNA or miRNA, since consumption of these RNAs by AGO proteins, present in different abundances along their route of movement, can restrict the distance they can reach (Devers *et al.*, 2020). Those endogenous siRNAs and miRNAs that are mobile often travel through the phloem from the shoots to roots (Molnar *et al.*, 2010; Li *et al.*, 2021), exploiting the flow of water transporting the photosynthates (Chen & Rechavi, 2022). That the phloem sap contains many miRNA species indeed suggests that miRNAs may travel through the phloem to distant tissues (Yoo *et al.*, 2004; Buhtz *et al.*, 2008). Though the long-distance movement of miRNAs occurs mostly through the phloem, miRNAs and siRNAs can move short distances via plasmodesmata (Vatén *et al.*, 2011; Rosas-Diaz *et al.*, 2018). There are several well-described miRNA modules that rely on the systemic transport of miRNAs to exert their physiological effects. In plants deprived of phosphate, the low phosphorus level detected in leaves triggers the expression of *miR399* genes, leading to the production of this miRNA. Subsequently, miR399 is translocated to the root system via the phloematic route. In the roots, miR399 represses its target mRNA, namely *PHO2*, a repressor of phosphate uptake. As a consequence, plants activate phosphate uptake from the soil (Bari *et al.*, 2006).

Another miRNA transported via the phloem is miR156, whose mobility was demonstrated as a graft-transmissible miRNA (Bhogale *et al.*, 2014). miR156is involved in controlling several developmental traits, such as juvenile-to-adult transition by regulating the expression of SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors (Wu *et al.*, 2009).

The systemic trafficking of sRNAs has also been demonstrated using a heterograft system of soybeans and common beans (Li *et al.*, 2021). Mobile sRNAs produced in the shoots are transported to the roots where they accumulate and regulate gene expression. These experiments demonstrated how sRNAs produced in the shoots of one species could be detected in the grafted rootstock of another species.

Although there is a lot of evidence of shoot-to-roots, less is known regarding root-to-shoot mobile miRNAs. Using an *Arabidopsis* / *Nicotiana* interfamilial heterograft system, through an sRNA deep sequencing analysis, it was demonstrated that a subset of miRNAs could travel from the roots to the shoots (Deng *et al.*, 2021).

III. MicroRNA mobility in plant–pathogen interactions

Apart from miRNA trafficking within the plant, recent evidence suggests a novel mechanism of communication between plants and their pathogens to induce gene silencing (Fig. 1). In this mechanism, known as cross-kingdom RNAi, pathogens deliver sRNAs into host plants to silence host immune response genes and, in turn, host plant cells send sRNAs in exosome-like extracellular vesicles into pathogens in order to silence virulence-related genes (Cai *et al.*, 2018b).

Cross-kingdom RNAi was initially observed during infection by the fungal pathogen *Botrytis cinerea*, the causal agent of grey mold disease in several crop plant species. sRNAs produced by *B. cinerea* can bind to Arabidopsis AGO1, thereby inducing gene silencing. AGO1, a member of the ARGONAUTE family, participates in the formation of the RNA-induced silencing complex, which directs RNAi by using the miRNA sequence as a guide to recognize which target RNA to degrade (Weiberg *et al.*, 2013).

Another study has shown that, in response to infection with the fungal pathogen *Verticillium dahliae*, cotton plants produce miR159 and miR166 and both miRNAs target fungal genes that are indispensable for virulence (Zhang *et al.*, 2016).

Cross-kingdom RNAi between plant and fungal pathogens is bidirectional. The sRNAs produced by the fungal pathogen are delivered into host cells to use the host AGO protein to suppress host defense genes. Conversely, the host-derived sRNAs through extracellular vesicles, especially exosomes, are transported into pathogens to suppress pathogen genes (Cai *et al.*, 2018b).

sRNAs also play a pivotal role during bacterial infection. It was suggested in a preprint paper that, in bacteria, sRNAs can pass through the host plasma membrane, as well as the complex multilayered bacterial cell envelope, and trigger gene silencing in the bacterium itself as part of an antibacterial defense response (Singla-Rastogi *et al.*, 2019). This trans-kingdom regulatory process is known as antibacterial gene silencing.

The discovery of host-induced gene silencing (HIGS) has led to the development of innovative tools for plant disease control. In HIGS approaches, genetically engineered plants express pathogen/pest gene-targeting sRNAs or dsRNAs. Taking advantage of cross-kingdom RNA trafficking, these sRNAs are transported into the

New Phytologist

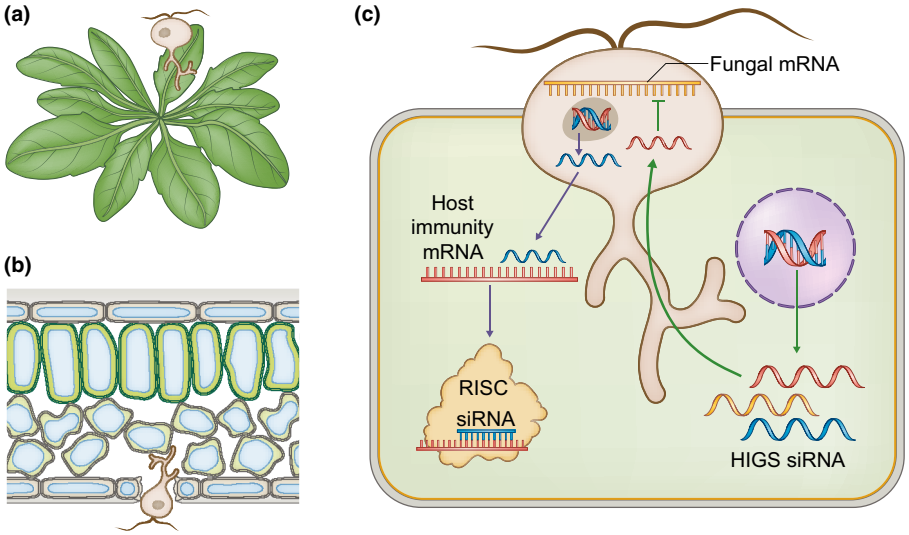

Fig. 1 Cross-kingdom RNA interference (RNAi) in plant–pathogen interactions. (a) Pathogens infect plants by attaching themselves to the plant's leaf or root systems. (b, c) Some pathogens enter though the stomata (b); however, an intimate relationship between the pathogen and the plant cell is then established (c), enabling the exchange of regulatory molecules. Among the signals exchanged by the plant and the pathogen are small RNAs (sRNAs). The pathogen delivers sRNAs into host plant cells (purple arrows), thus suppressing the host immune responses. This occurs by silencing the host immunity-related messenger RNA (mRNA) by using the host cell RNAi machinery. Plant cells also deliver sRNAs (host-induced gene silencing (HIGS) small interfering RNA (siRNA)) into pathogen cells (green arrows). RISC, RNA-induced silencing complex.

pathogen or pest in order to silence virulence genes, thus increasing plant tolerance to disease. Various plant species have been engineered, from model plants to commercial crops, aimed at combating pathogens such as nematodes, viroids, viruses, insects, and fungi (Niu *et al.*, 2021).

Interestingly, in approaches that avoid the use of transgenic plants, it was demonstrated that the direct application of the RNA spray, named spray-induced gene silencing (SIGS), of pathogen-gene-targeting RNAs confers resistance to the pathogen (Cai *et al.*, 2018a). In SIGS technologies, chemically synthetized dsRNAs or sRNAs are used. The success of SIGS depends on the efficiency of RNA uptake by the pathogen, with higher degrees of uptake of dsRNA correlating with a higher protection against the pathogen (Qiao *et al.*, 2021).

In order to increase plant protection against plant pathogens, layered double hydroxide (LDH) clay nanosheets complexed with the exogenous RNA represent an effective delivery method that could protect plants from virus attack for 20 d after a single spay instead of the 5–8-day protection provided by naked dsRNA or sRNA sprays on plants. LDH appears to act by enhancing the stability of the otherwise labile naked dsRNA (Mitter *et al.*, 2017).

IV. MicroRNAs as plant-to-plant signaling molecules

Naturally occurring trans-species miRNA trafficking has been described between parasitic plants and their host (Fig. 2). The parasitic plant *Cuscuta* uses haustoria to get water and nutrients from its host plant. In *Cuscuta campetris*, a high number of 22-nt-long miRNAs are induced at the haustorium when it parasitizes Arabidopsis and tobacco (Shahid *et al.*, 2018). These miRNAs can hijack the silencing machinery of host plants in order to induce the production of secondary siRNAs and the subsequent degradation of host mRNAs (Shahid *et al.*, 2018).

The fact that some miRNAs are mobile molecules within the plant, and that they can travel between the plant and their pathogen and between parasitic plants and host plants, suggests miRNAs may play a role in plant-to-plant communication (Fig. 3). Recent reports (Betti *et al.*, 2021; Marzec, 2022) described the first evidence of miRNA communication between plants, with experimental evidence that plants can take up miRNAs produced by the neighboring plants, thereby inducing PTGS in the receiving plant. The evidence of plant-to-plant miRNA transfer was reported in the well-known miR399/PHO2 and miR156/SPL modules (Betti *et al.*, 2021). Both these miRNAs are known to be cell mobile (Pant *et al.*, 2008; Bhogale *et al.*, 2014), and the physiological processes they regulate have been well characterized.

In the Betti *et al.* study, Arabidopsis seedlings were fed with a plant extract obtained from miRNA-overexpressing (either miR399 or miR156) plants. These extracts were thus enriched in one specific miRNA, hence enabling the response to be tested in a plant with a basal expression of the miRNA studied. Interestingly, wild-type Arabidopsis seedlings displayed downregulation of miR399/miR156 targets when treated with the exogenous miRNAs-containing extract, thus suggesting that plants can take up miRNAs from the medium, with PTGS downregulating their target genes. These exogenous miRNAs can be both 'natural' (extracted from a plant and fed as a mixture of RNAs) or chemically synthesized. The uptake of miRNAs occurs preferentially though the roots, which are then transported through the xylem (Betti *et al.*, 2021). This was highlighted using fluorophore-labeled miR399, thus showing that a chemically synthetized, pure miRNA can enter the vascular tissue and move to distal tissue. Along with

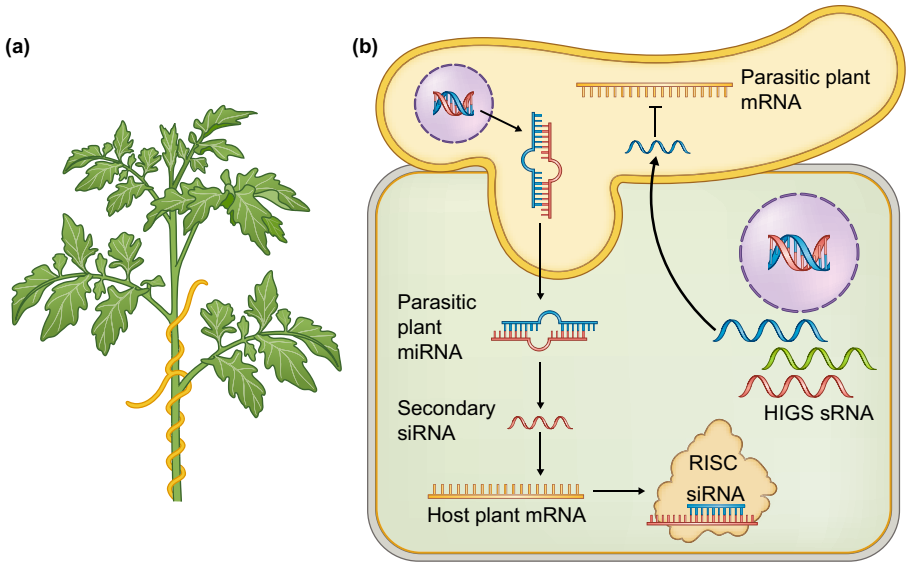

Fig. 2 Interspecies small RNA (sRNA) trafficking between parasitic plants and their host. (a) Parasitic plants such as dodder (*Cuscuta campestris*, in yellow in the figure) are obligate parasitic plants. They use specialized feeding structures called haustoria to obtain water and nutrients from the stems of host plants. The haustoria can also facilitate the bidirectional movement of RNAs acting as regulatory molecules. (b) *Cuscuta campestris* produces specific 22-nt microRNAs (miRNAs) that are transferred to the host plant cells where they silence host target genes involved in the host defense by RNA interference mediated by the production of secondary small interfering RNAs (siRNAs). The cells of the host plant produce host-induced gene silencing (HIGS) sRNAs to target the parasitic messenger RNAs (mRNAs).

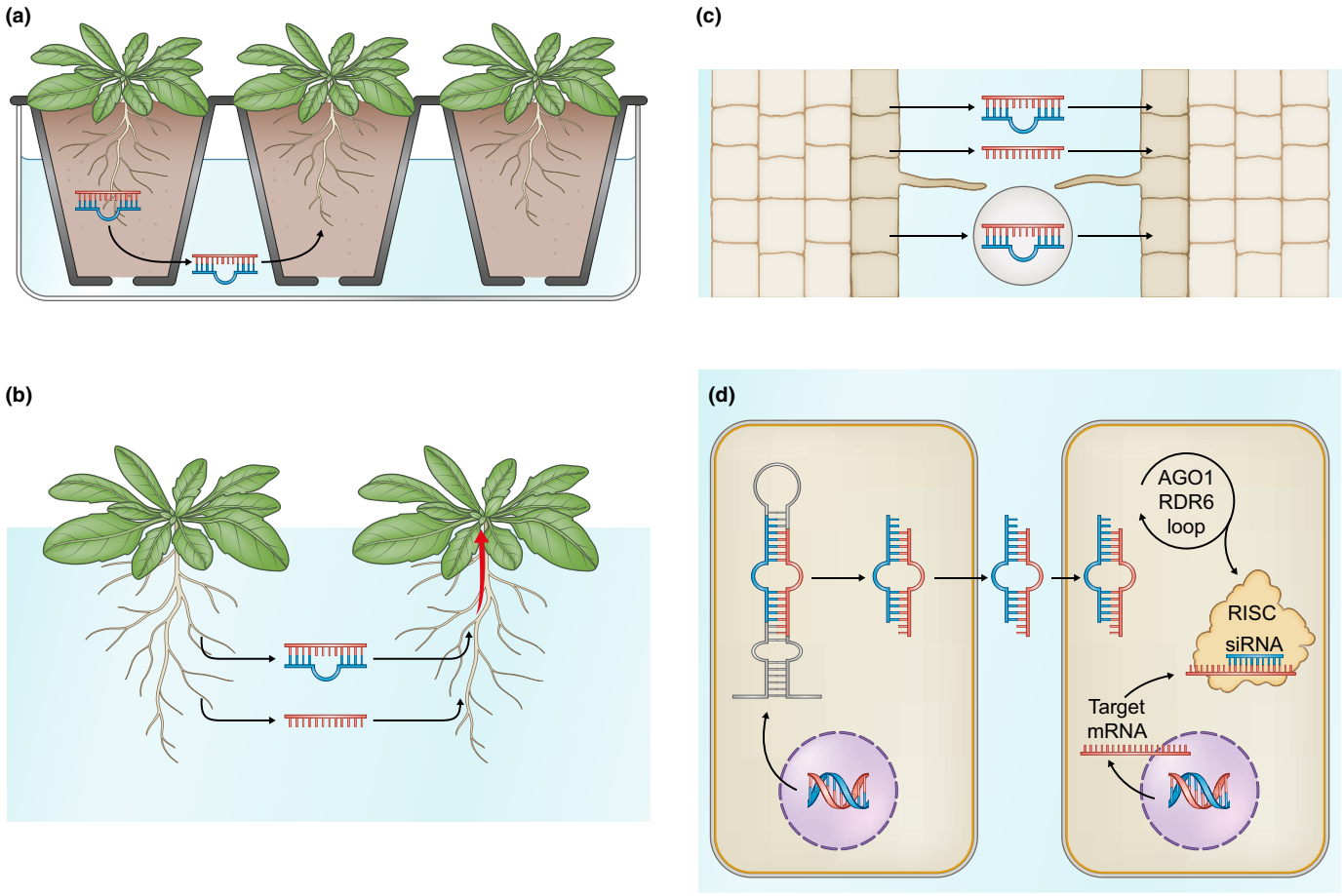

Fig. 3 MicroRNAs (miRNAs) act as plant-to-plant regulatory molecules. (a) When Arabidopsis plants are grown in a tray sharing the same growth medium, miRNAs are secreted into the medium and influence the phenotype of the nearby receiving plants. (b) miRNAs, secreted as duplexes or single-stranded mature miRNAs, are taken up by the receiving plant roots and translocated into the plant via the xylematic route (red arrow). (c) It is still unclear which is the preferential form by which miRNAs are secreted and taken up. The possible forms include the miRNA duplex, the single-stranded mature strand, and exosomes containing the miRNA molecules. (d) miRNAs are produced in the donor plant, where they are processed from the primary miRNA to the mature miRNA, which is secreted following an unknown mechanism to the external medium. The root cells of nearby plants can take up these exogenous miRNAs, which induce signal amplification by a loop requiring ARGONAUTE1 (AGO1) and RNA-dependent RNA polymerase 6 (RDR6) producing secondary small interfering RNAs (siRNAs) that silence the target gene(s) in the receiving plant. mRNA, messenger RNA; RISC, RNA-induced silencing complex.

other evidence, this suggests that the uptake of exogenous miRNAs by plant roots does not require delivery by exosomes.

The consequence of exogenous RNA uptake on the regulation of gene expression should also impact on the plant's phenotype. Exploiting the well-known reduced primary root phenotype in the transgenic line overexpressing miR156a (Barrera-Rojas *et al.*, 2020), it was demonstrated that exogenous miR156 inhibited primary root growth in a vertical agar-plate assay. Other evidence supports the role of miRNAs as signaling molecules operating between different plants. First, exogenous miRNAs were observed in an exogenous hydroponic medium. This is important, because it suggests that (1) plants secrete miRNAs to the outside medium and (2) miRNAs are stable in a nonsterile environment. When wild-type and miRNA (miR399/miR156)-overexpressing plants were co-cultivated in the same hydroponic tray, the expression level of *PHO2/SPL*s genes decreased in wild-type plants, thus demonstrating that the higher level of miRNAs in the transgenic overexpressor influenced the expression of the relative miRNA target gene in a nearby wild-type plant. Interestingly, the flowering of wild-type plants is delayed when co-cultivated with an miR156 overexpressor, which is a genotype with extremely delayed flowering (Betti *et al.*, 2021).

In plants, RNAi triggered by most miRNAs requires the AGO1 protein, although there are reports suggesting a role of AGO8 in *Nicotiana attenuata* (Pradhan *et al.*, 2017) and AGO4 during plant pathogen interaction (Pradhan *et al.*, 2020). In Arabidopsis, an AGO1 mutant was shown to be nonresponsive to exogenous miRNAs (Betti *et al.*, 2021), and an *rdr6* mutant was also insensitive to exogenous miRNAs. Taken together, these results indicate that exogenous miRNAs enter the canonical pathways of PTGS with RDR6-dependent transitivity.

The ability of exogenous miRNAs to induce PTGS in the receiving plant expands the concept of miRNA mobility well beyond the *in planta* translocation of RNA-based signals. The exchange of miRNAs between plants and pathogens/parasitic plants has already highlighted the potential role of miRNAs in communication between distinct living organisms, but in both cases there is a very close contact between the cells of producing/receiving plants.

V. Conclusions

The exchange of miRNAs between separate plants growing nearby has a number of implications and raises new questions. First, it is unclear how miRNAs are secreted from plants to the outside medium. The most logical explanation is that exosomes represent the mechanism by which miRNAs exit the plant cell, as clearly shown in the plant–pathogen exchange of RNAs (Cai *et al.*, 2018b). However, apoplastic miRNAs are not always inside exosomes (Zand Karimi *et al.*, 2022). Another possibility is that miRNAs leak out of decaying roots into the soil. Once in the external medium, miRNAs are clearly very stable. This could be due to their double-stranded structure; however, the ratio between the mature miRNA strand and the passenger strand is largely in favor of the mature strand in plant extracts (Betti *et al.*, 2021), raising a question related to the nature of the structure of the miRNAs released by the plant to the outside medium. Given that most of the guide strand of miRNAs in plants is loaded on the AGO protein(s), it is possible that miRNAs loaded on AGO proteins are mobile or that the miRNA duplex, rather than the single strand, is the mobile version of miRNAs.

Transitivity by RDR6 is required for PTGS by exogenous miRNAs. This is compatible with *miR156* and *miR399* both being secondary siRNA-triggering miRNAs (Manavella *et al.*, 2012) because of their miRNA duplex structure. miR156 and miR399 both have a 21 nt mature strand but a 22 nt passenger strand, which is required for inducing transitivity (Manavella *et al.*, 2012). Though the duplex was fed when a chemically synthesized pure miR156 or miR399 was used, feeding with an RNA extract was predominantly composed of the mature strand (Betti *et al.*, 2021). This suggests that either the relatively small number of passenger miRNAs that was exogenously fed is sufficient for triggering RDR6-dependent production of secondary siRNAs, or that the mature miRNA is sufficient as an exogenous regulatory molecule, possibly by association with the passenger miRNA produced by the receiving plant.

How the receiving plants can internalize exogenous miRNAs is unknown. Exosomes do not seem to be required, given that PTGS was induced by naked, pure miRNAs (Betti *et al.*, 2021). However, it is also possible that exosomes are involved in the *in vivo* exchange of miRNAs between plants. However, the regulatory activity by naked miRNAs indicates that plants have the ability to take up exogenous RNAs. This is compatible with the multiple evidence of PTGS triggered by SIGS, when exogenous, naked dsRNAs are applied exogenously onto the plant (Wang & Jin, 2017). Nematodes possess SID-1, a dsRNA-selective dsRNA-gated channel, allowing environmental RNAi (Shih & Hunter, 2011). The existence of a channel allowing miRNA uptake in plants is unknown, and its discovery would be a massive breakthrough in plant RNA biology.

Are all miRNAs able to trigger PTGS when exogenously applied? Given the evidence of RDR6 being required for PTGS by exogenous miRNAs, it is likely that only miRNAs inducing secondary siRNA production (Manavella *et al.*, 2012) represent molecules able to allow plant-to-plant communication.

Translocation by the xylem seems to be the preferential route for root-fed exogenous miRNAs (Betti *et al.*, 2021). This is in contrast to several sources of evidence of preferential phloem movement of miRNAs in plants (Kehr & Buhtz, 2008). However, the presence and translocation of RNAs restricted to the xylem was observed for hairpin RNA and sRNAs after trunk injection and petiole absorption (Dalakouras *et al.*, 2018). The route followed by miRNAs in the plant may, therefore, depend on the site of entry of the RNA, with the phloem involved in the in-plant RNA movement and the xylematic route preferentially used when the source of RNA is exogenous.

All these questions await answers from experimental evidence. The field of exogenous RNA biology in plants has already provided outstanding breakthroughs in cross-species communication and plant disease control (Cai *et al.*, 2019). It is now possible to explore the importance of exogenous miRNAs present in the soil for signaling in plant communities.

Acknowledgements

Open access funding provided by Consiglio Nazionale delle Ricerche within the CRUI-CARE agreement.

Author contributions

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