New Phytologist

Review

Research review

A journey into the world of small RNAs in the arbuscular mycorrhizal symbiosis

Authors for correspondence:
Luisa Lanfranco
Email: luisa.lanfranco@unito.it

Ignacio Rubio-Somoza
Email: ignacio.rubio@cragenomica.es

Received: *31 July 2023*
Accepted: *15 October 2023*

William Conrad Ledford[1,2], Alessandro Silvestri[2], Valentina Fiorilli[1], Ronelle Roth[3], Ignacio Rubio-Somoza[2,4] and Luisa Lanfranco[1]

[1]Department of Life Sciences and Systems Biology, University of Turin, Turin, 10125, Italy; [2]Molecular Reprogramming and Evolution (MoRE) Lab, Centre for Research in Agricultural Genomics (CRAG), CSIC-IRTA-UAB-UB, Barcelona, 08193, Spain; [3]Department of Biology, University of Oxford, Oxford, OX1 3RB, UK; [4]Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, 08001, Spain

New Phytologist (2023)
doi: 10.1111/nph.19394

Key words: arbuscular mycorrhizal symbiosis, cross-kingdom RNA interference, plant–microbe interactions, RNA interference, small RNA.

Summary

Arbuscular mycorrhizal (AM) symbiosis is a mutualistic interaction between fungi and most land plants that is underpinned by a bidirectional exchange of nutrients. AM development is a tightly regulated process that encompasses molecular communication for reciprocal recognition, fungal accommodation in root tissues and activation of symbiotic function. As such, a complex network of transcriptional regulation and molecular signaling underlies the cellular and metabolic reprogramming of host cells upon AM fungal colonization. In addition to transcription factors, small RNAs (sRNAs) are emerging as important regulators embedded in the gene network that orchestrates AM development. In addition to controlling cell-autonomous processes, plant sRNAs also function as mobile signals capable of moving to different organs and even to different plants or organisms that interact with plants. AM fungi also produce sRNAs; however, their function in the AM symbiosis remains largely unknown. Here, we discuss the contribution of host sRNAs in the development of AM symbiosis by considering their role in the transcriptional reprogramming of AM fungal colonized cells. We also describe the characteristics of AM fungal-derived sRNAs and emerging evidence for the bidirectional transfer of functional sRNAs between the two partners to mutually modulate gene expression and control the symbiosis.

Introduction

Arbuscular mycorrhizal (AM) symbiosis is an intimate interaction between the roots of most land plants and soil fungi of the subphylum Glomeromycotina. This mutualistic interaction, based upon the trading of mineral nutrients (mainly phosphates) from fungi to plants in exchange for carbon compounds, dates back to the Devonian when it was pivotal for plant terrestrial colonization. Thus, such a relationship implies a long history of plant–fungus co-evolution and a finely tuned coordination of developmental and metabolic processes in both partners (reviewed in Genre *et al.*, 2020).

During the presymbiotic phase, plant and AM fungi release diffusible molecules that enable mutual recognition and induce reciprocal symbiotic responses: Host plants attract and stimulate the fungal partner through the release of strigolactones (SLs) and perhaps other still uncharacterized molecules. In AM fungi, the perception of SLs increases the production of short chitin-based oligomers (chito-oligosaccharides and lipo-chito-oligosaccharides) generally referred to as Myc factors, which, in turn, enhance plant SLs production (Volpe *et al.*, 2023) and induce plant responses essential for fungal accommodation. Myc factors activate the genetic program known as the Common Symbiotic Signalling Pathway (CSSP) that largely shapes the transcriptional response to AM colonization and nodulation in legume plants (Oldroyd, 2013; MacLean *et al.*, 2017; Pimprikar & Gutjahr, 2018).

Once inside plant roots, AM fungi develop intercellular hyphae and highly branched intracellular structures within the inner cortex layers, called arbuscules. In arbuscule-containing cells, the plant cell plasma membrane extensively envelops even the finest fungal hyphae, leading to the formation of the peri-arbuscular membrane (PAM), which presents a characteristic lipid and

protein composition (Ivanov & Harrison, 2018; Roth *et al.*, 2018). Between the arbuscule/fungal cell membrane and the PAM spans an apoplastic zone, called the peri-arbuscular space (PAS), where a bidirectional transport of nutrients and, possibly, several signaling molecules from fungal to plant cells and vice versa occurs. Although little is known about the types of molecules and the mechanisms involved, these exchanges likely play a key role in the regulation of the symbiosis.

Although fungal growth is restricted to roots, AM colonization also leads to metabolic and physiological changes at the systemic level, according to the occurrence of a root-shoot axis influencing multiple aspects of plant biology including nutrition, development and responses to (a)biotic stresses (Fiorilli *et al.*, 2018; Chialva *et al.*, 2023).

Local, as well as systemic responses, upon AM colonization imply a fine-tuning of plant and fungal gene expression that is crucial for the establishment and functioning of the mutualistic association. Accordingly, many studies have reported extensive and conserved transcriptional reprogramming across different plant species in response to AM fungi, including genes present only in the genomes of AM-competent species (Delaux *et al.*, 2014; Bravo *et al.*, 2016). Besides transcription factors and *cis*-regulatory elements (Pimprikar & Gutjahr, 2018), small RNAs (sRNAs) have also been deemed important regulators embedded in the genetic networks orchestrating AM development (Lauressergues *et al.*, 2012; Bazin *et al.*, 2013; Etemadi *et al.*, 2014; Couzigou *et al.*, 2017; Müller & Harrison, 2019; Pradhan & Requena, 2022). Likewise, AM fungi undergo profound reprogramming that might include sRNAs since they possess all the essential components of RNA interference (RNAi) machinery regulating the production of sRNA from different genetic sources (Silvestri *et al.*, 2019, 2020; Dallaire *et al.*, 2021; Manley *et al.*, 2023).

In addition to controlling cell-autonomous processes, sRNAs may also function as mobile signals within the plant (Li *et al.*, 2021), and even between different plants (Shahid *et al.*, 2018; Betti *et al.*, 2021) and the microorganisms they interact with, in a process known as cross-kingdom RNA interference (ckRNAi, Cai *et al.*, 2018a). Accordingly, plants impaired in the production of sRNAs show dysbiosis (Kaushal *et al.*, 2021).

In this review, we aim to summarize known sRNA pathways and discuss the contribution of sRNAs in the establishment and functioning of AM symbiosis, examining the reprogramming of plant sRNAs upon colonization by AM fungi and the landscape and significance of sRNAs in the fungal symbionts; lastly, we comment on emerging evidences for ckRNAi involving the possible transfer of functional sRNAs between the two symbiotic partners.

The RNA interference machinery: a flexible platform based on multiple components and a plethora of small RNA types

RNA interference (RNAi) is a universal gene regulatory system in eukaryotes that orchestrates development and stress responses. This regulatory system is based on sequence complementarity between sRNAs and their DNA (transcriptional gene silencing, TGS) or RNA (post-transcriptional gene silencing, PTGS) targets. Plant sRNAs typically range from 20 to 28 nucleotides in length and belong to two different subclasses according to the RNA template they originate from via the action of RNAse-like II enzymes from the DICER-Like family (DCL). Micro RNAs (miRNAs) are produced from single-stranded RNAs (ssRNAs), transcribed from endogenous genes, that self-fold into highly thermodynamically stable hairpins, which are then processed into mature miRNAs mainly by the action of DCL1. Plant miRNAs are involved in PTGS by triggering the cleavage of their mRNA targets or by inhibiting their translation. Short interfering RNAs (siRNAs) constitute the second class of sRNAs and are produced from fully complementary double-stranded RNAs (dsRNAs) of exogenous and endogenous origin. siRNA participates both in PTGS (mediated by 21–22 nt siRNAs) and in TGS through the RNA-directed DNA methylation pathway, which is mediated by 24 nt heterochromatic-siRNAs (hc-siRNAs). Most endogenous siRNA precursors originate from ssRNA templates that are converted into dsRNAs by enzymes from the RNA-dependent RNA polymerase family (RdRps). Processing of those dsRNAs into siRNAs occurs in a phased manner (pha-siRNAs). After their biogenesis, the resulting sRNA duplexes are further methylated and stabilized by the conserved HUA ENHANCER1 enzyme (HEN1, Yu *et al.*, 2005; Sanobar *et al.*, 2021) and loaded into the RNA-induced silencing complex (RISC). Proteins from the ARGONAUTE (AGO) family are the main effectors forming the RISC. Eukaryotic AGO proteins share many common features and functions through conserved RNAi pathways. However, plant AGOs vary largely in number among species and have diversified into three major phylogenetic clades, as exemplified in the model *Arabidopsis thaliana* by AGO1/5/10, AGO2/3/7 and AGO4/6/8/9 (Li *et al.*, 2022; Bélanger *et al.*, 2023). Sorting of sRNAs onto AGOs is a regulated process, where the length (Mi *et al.*, 2008; Takeda *et al.*, 2008), the 5′ nucleotide (Mi *et al.*, 2008) and the secondary structure of the duplexes act as determinants for differential AGO loading and subsequent function. Interestingly, the regulatory function of sRNA-loaded AGO proteins extends beyond the cells in which they are primarily expressed. This regulatory function can occur through their inclusion in extracellular vesicles (EVs; He *et al.*, 2021) or by secretion into the apoplastic fluid along with long noncoding and circular RNAs (Zand Karimi *et al.*, 2022) that have been proposed to act in mammals as miRNA sponges (Olesen *et al.*, 2021).

It is important to note that most of what is known about DCL-dependent biogenesis of sRNAs, sorting into AGO proteins and their functions in plants is based on the model plant species *Arabidopsis thaliana*, a non-host plant for AM fungi. Other plant species may present additional gene family members with specific expression patterns, subcellular location or specialized functions arising from gene duplication and sub- and/or neofunctionalization. Examples of functional specialization include the monocot-specific DCL5, which evolved from the duplication of an ancient DCL3 from a eudicot ancestor (Ono *et al.*, 2018) and that is involved in the cell-type specific production of 24-nt sRNAs that participate in temperature-dependent fertility (Teng *et al.*, 2020). Another example is the preferential loading of unusually long

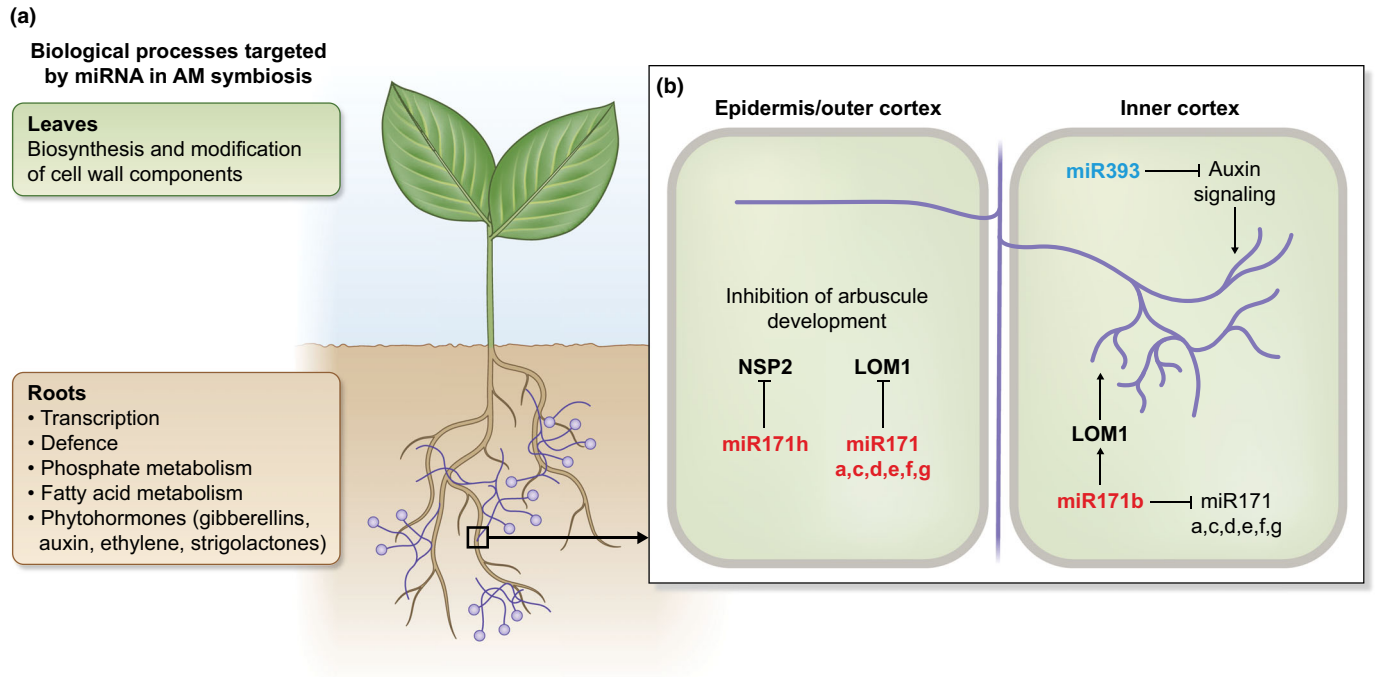

Fig. 1 Micro RNA (MiRNA)-mediated plant gene expression reprogramming in the arbuscular mycorrhizal (AM) symbiosis. (a) SmallRNAome survey in different plant species identified target genes involved in several biological processes (Gu *et al*., 2010; Devers *et al*., 2011; Formey *et al*., 2014; Wu *et al*., 2016; Pandey *et al*., 2018; Mewalal *et al*., 2019; Mendoza-Soto *et al*., 2022; Zeng *et al*., 2023). (b) Specific miRNA-target interactions were shown to control the AM colonization. The downregulation (blue color) of miR393 by AM symbiosis releases its suppressive effect on members of the auxin signaling pathway, thereby promoting arbuscule development (Etemadi *et al*., 2014). miR171h, which is expressed in epidermis and outer cortex layers, by targeting the NSP2 transcription factor, prevents AM over-colonization (Lauressergues *et al*., 2012). miR171b is induced (red color) in arbuscule-containing cells where it protects LOM1 from being targeted by other members of the miR171 family (miR171a,c,d,e,f,g), promoting arbuscule formation (Couzigou *et al*., 2017).

sRNAs (> 26 nt) into AGO1 in the unicellular alga *Chlamydomonas reinhardtii* (Li *et al*., 2023).

Plant sRNAs shape plant-biotic interactions including the AM symbiosis

Besides having a central role in regulating endogenous gene expression, RNAi safeguards genome integrity from transposable elements (TEs) and constitutes one of the main antiviral defense mechanisms. During their life cycle viruses produce highly stable dsRNAs intermediates that are recognized and processed by the host RNAi machinery, generating viral small RNAs (vsRNAs) that are used to seek and destroy viral genomes and stop the infection. In addition, vsRNAs can also target host transcripts, promoting viral infection (Smith *et al*., 2011). Perception of molecules derived from bacterial and fungal pathogens, such as flagellin and chitin, also leads to changes in the levels of several plant sRNAs which, in turn, can elicit antimicrobial defense responses (Navarro *et al*., 2006; Li *et al*., 2010; Boccara *et al*., 2014; Soto-Suárez *et al*., 2017; López-Márquez *et al*., 2021; Vasseur *et al*., 2022).

Notably, the role of plant sRNAs in plant–microbe interactions is not restricted to their direct regulation of endogenous plant immune programs but it can be exerted distantly through their translocation to the pathogenic microorganism. Since the finding that miR159 and miR166 from cotton were targeting virulence genes in the fungal pathogen *Verticillium dahlia* abrogating infection (Zhang *et al*., 2016), other studies have shown an antimicrobial role of plant-derived sRNAs (Cai *et al*., 2018b; Hou *et al*., 2019; Zhu *et al*., 2022).

While there is a wealth of literature on the role of sRNAs in plant-pathogenic interactions, the current knowledge on the sRNA-mediated gene regulation network controlling the AM symbiosis is still limited and possibly represents the tip of the iceberg. The colonization by AM fungi induces an extensive reprogramming of plant sRNAs expression, many of which are conserved in different plant species (Gu *et al*., 2010; Devers *et al*., 2011; Formey *et al*., 2014; Wu *et al*., 2016; Pandey *et al*., 2018; Xu *et al*., 2018; Mewalal *et al*., 2019; Mendoza-Soto *et al*., 2022; Zeng *et al*., 2023; Fig. 1). Upon AM fungal colonization in *Medicago truncatula*, a number of miRNAs are transcriptionally upregulated with predicted miRNA targets mostly represented by transcription factors that modulate AM development and defense-related genes to suppress immunity (Devers *et al*., 2011). Similar results were obtained in the host plant *Nicotiana attenuata* (Pandey *et al*., 2018). In this case, a specific member (AGO7) of the AGO family was shown to contribute to the AM-induced sRNA modulation that allowed to control of root colonization levels through the actions of various phytohormone signaling pathways (gibberellins, ethylene and auxin) and phosphate and fatty acid metabolism (Pradhan *et al*., 2023). Moreover, the downregulation of *N. attenuata* AGO7 by RNAi led to an increased mycorrhization level (Pradhan *et al*., 2023). Interestingly, enhanced root nodulation was described in a *M. truncatula* AGO7

mutant (Hobecker *et al.*, 2017), suggesting that shared regulatory processes may occur in these two root symbiotic interactions, possibly crossing the CCSP.

The underlying mechanisms of plant miRNA-mediated gene regulation in mycorrhizal roots have been characterized in only a few cases. Members of the miR399 family, systemic inorganic phosphate (Pi)-starvation signals that suppress the E2 conjugase *Phosphate 2* (*PHO2*) needed to maintain Pi homeostasis, have been proposed as root-to-shoot signaling molecules in the regulation of the AM symbiosis by Pi. The expression of members of the miR399 family is indeed modulated in mycorrhizal plants (Branscheid *et al.*, 2010; Pandey *et al.*, 2018; Xu *et al.*, 2018; Zeng *et al.*, 2023). However, miR399 overexpression did not restore AM fungal colonization at high Pi concentration (Branscheid *et al.*, 2010), suggesting that other mechanisms are involved. Recent findings provided evidence that a plant regulatory network centered on crucial components of the so-called Pi starvation response (PHR) controls the level of AM root colonization (Shi *et al.*, 2021; Das *et al.*, 2022).

Opposite to what has been found in defense against bacterial (Navarro *et al.*, 2006) and fungal pathogens (Shi *et al.*, 2021), an increase in core elements under miRNA regulation from the auxin signaling pathway has a positive role in regulating symbiotic interactions, including the AM colonization. Thus, it has been shown that miR393 is downregulated in mycorrhizal roots, suggesting that TIR1/AFB-dependent auxin signaling is required for arbuscule formation (Etemadi *et al.*, 2014; Fig. 1).

In contrast to what was found in Arabidopsis, where higher levels of miR396b expression increased plants' susceptibility to fungal pathogens (Soto-Suárez *et al.*, 2017), miR396b overexpression in *M. truncatula* led to reduced mycorrhization, possibly mediated by the silencing of target transcription factors belonging to the GRF and bHLH families (Bazin *et al.*, 2013).

Interestingly, the expression of some plant AM-related genes is under the control of miRNAs (Müller & Harrison, 2019; Zeng *et al.*, 2023). The best characterized example concerns miRNAs of the conserved family miR171, known for regulating the expression of GRAS transcription factors – TFs (Cenci & Rouard, 2017). Notably, in *M. truncatula* it was reported that the isoform mtr-miR171h, targeting the NSP2 (Nodulation Signaling Pathway2; Fig. 1) GRAS TF, is specifically induced by AM fungi or treatments with symbiotic diffusible molecules such as Myc factors (Branscheid *et al.*, 2010; Devers *et al.*, 2011; Lauressergues *et al.*, 2012; Hofferek *et al.*, 2014). NSP2 cooperates with other GRAS TFs, such as NSP1 and RAM1 (Required for Arbuscular Mycorrhization-1) to promote the expression of AM-specific genes required for correct root colonization by AM fungi (Gobbato *et al.*, 2012; Rich *et al.*, 2017). In particular, mtr-miR171h is specifically expressed in those root peripheral tissues (e.g. epidermis and first cellular layer of the cortex of the elongation zone) in which the colonization or over-colonization by AM fungi should be prevented (Lauressergues *et al.*, 2012). Furthermore, the miR171 family is also known for controlling the expression of LOM (Lost Meristems) genes, another group of GRAS TFs (Xue *et al.*, 2015). In *M. truncatula*, LOM1 is a positive regulator of the AM symbiosis (Couzigou *et al.*, 2017; Fig. 1): the isoform mtr-miR171b, that is encoded only by genomes of AM-competent plants, is specifically induced in cells containing arbuscules and protects LOM1 from being targeted by other members of the miR171 family, thanks to a mismatch in the cleavage site (Couzigou *et al.*, 2017). The mismatch makes AGO-miR171b complex cleavage-incompetent toward LOM1 transcripts and, by competing for the same substrate with other cleavage-competent isoforms of miR171, miR171b prevents the silencing of its target. It has been hypothesized that this protective mechanism of action of miRNAs may exist in other plant miRNA families (Couzigou *et al.*, 2017; Fig. 1).

Although the majority of the investigations were performed on herbaceous species and on roots, more recently, attention was also given to woody plants and epigeous organs. The differential expression of 130 miRNAs was identified in *R. irregularis*-colonized roots of *Populus trichocarpa* (Mewalal *et al.*, 2019), among which were homologs of miRNAs shown to be AM-responsive in other species (miR156, miR160, miR170, miR167, miR393 and miR396; Bazin *et al.*, 2013; Etemadi *et al.*, 2014; Wu *et al.*, 2016). Moreover, 39 AM-responsive miRNAs showed a similar expression trend when poplar plants were colonized by the ectomycorrhizal fungus *Laccaria bicolor*. This indicates that the plant miRNA landscape and hence, the molecular mechanism controlled by this level of regulation, may be conserved to a certain extent across species and even across endo- and ectomycorrhizal types.

Systemic responses to mycorrhization, which might be linked to miRNA-regulated processes, have been found in leaves of AM-colonized plants. Differentially expressed miRNA/targets were identified in leaves of mycorrhizal tomato plants (Mendoza-Soto *et al.*, 2022). Among the identified miRNAs, miR164a-3p, miR164a-5p, miR171e-5p and miR397 target genes related to the biosynthesis or modification of cell wall components. These findings support the hypothesis that the remodeling of the plant cell wall participates in the induction by mycorrhizal colonization of a priming state, that confers increased tolerance against foliar pathogens (Mendoza-Soto *et al.*, 2022).

To have a full view of the sRNA-mediated plant gene regulation network occurring in the AM symbiosis, integrated analyses of the different populations of RNA, covering the sRNAs and their targets and including the so far little characterized tiny and circular RNAs, should be performed (Zeng *et al.*, 2023). The role of specific members from the DCL and/or AGO family in the AM symbiosis also deserves further investigation (Huang *et al.*, 2019; Pradhan & Requena, 2022; Pradhan *et al.*, 2023).

Insights on small RNA and RNAi in AM fungi

Almost all fungal lineages possess the key components of the RNAi machinery (Torres-Martínez & Ruiz-Vázquez, 2017; Lax *et al.*, 2020). Fungi often present more than one gene encoding for the basic RNAi components, typically 1–2 DCL, 1–4 AGO and 1–4 RdRp (Chang *et al.*, 2012). AM fungi are characterized by a conserved repertoire of core RNAi genes that exhibit distinctive attributes from other fungi (Lee *et al.*, 2018; Silvestri *et al.*, 2019, 2020; Dallaire *et al.*, 2021; Lanfranco & Bonfante, 2023). A first salient hallmark of AM fungi is the expansion of the *AGO* gene family: In *R. irregularis*, this gene family consists of 26 complete

AGO genes and an additional 14 genes that contain the typical PIWI domain but lack other AGO domains. In the *Rhizophagus* genus, the RdRP gene family also shows some level of expansion, with *R. irregularis* having 21 RdRps, six of which correspond to small peptides containing a short RdRp domain unreported in other species (Silvestri *et al.*, 2019). AM fungi possess 1 or 2 DCLs but, remarkably, contain uniquely in eukaryotes, prokaryotic-type class I ribonuclease III homologs arising from horizontal gene transfer (HGT) events with cyanobacteria that may contribute to the production of sRNAs (Lee *et al.*, 2018). AM fungi have maintained the sRNA methyltransferase HEN1, which is known to confer higher stability to plant sRNAs, while this gene was lost in several genomes of other mycorrhizal (ectomycorrhizal, orchid and ericoid) and non mycorrhizal plant-interacting fungi (Dallaire *et al.*, 2021). Transcriptomics and proteomic data show these RNAi-related genes are expressed and sometimes regulated in different stages of the fungal life cycle (Silvestri *et al.*, 2019, 2020; Dallaire *et al.*, 2021).

As expected by the presence of a complete RNAi machinery, AM fungi produce sRNAs with typical hallmarks of RNAi-equipped organisms, as demonstrated by a few sRNA-Seq experiments (Silvestri *et al.*, 2019, 2020; Dallaire *et al.*, 2021). These studies showed that the sRNA reads had a unimodal length distribution centered at 24, 25, or 26 nucleotides depending on the species (*R. irregularis* and *G. margarita*) and the fungal stage analyzed. This length distribution is expected from species possessing a single DCL. The sRNA reads also showed enrichment in uracyl at their 5′-end, a common feature of RNAi-competent fungi. In *R. irregularis*, Dallaire *et al.* (2021) also demonstrated that a significant subset of sRNAs is 2′-O-methylated at the 3′-end, a modification likely provided by HEN1 homologs. Despite the available data being limited to only two species (*G. margarita* and *R. irregularis*), comprehensive sRNA genomic annotations revealed the existence of two distinct populations of sRNA-generating loci in AM fungi (Silvestri *et al.*, 2019, 2020). The first group of loci localizes on unannotated regions or overlaps with TEs, producing typical RNAi-related sRNAs in length (22-to-26 nt-long sRNAs). The second group of loci originates from protein-coding genes and produces sRNAs not enriched in a specific length. A noncanonical DCL-independent pathway (NCRIP) that generates sRNAs with similar features has been described in the phylogenetically related nonmycorrhizal fungus *Mucor circinelloides* (Trieu *et al.*, 2015; Pérez-Arques *et al.*, 2020). Interestingly, this pathway depends on a prokaryotic-derived RNAse III protein (R3B2), likely a product of HGT between a bacterium and a *Mucorales* ancestor. This evidence also suggests that the AM fungi-specific prokaryotic-type ribonuclease III may contribute to sRNA production with an NCRIP. Moreover, a few sRNA-generating loci have been annotated as miRNA-like (Silvestri *et al.*, 2019, 2020).

The recent re-annotation of TEs and the characterization of their transcriptional and epigenomic dynamics in *R. irregularis* demonstrated that both RNAi and 5-methylcytosine at CG sites play a role in controlling TEs (Dallaire *et al.*, 2021). TE activity may be crucial for generating adaptive genetic variation in asexual organisms such as AM fungi, but it also poses a risk to genome integrity. In this context, a finely tuned anti-TE AGO-mediated defense activity seems essential to balance the potential benefits (genome variability) and drawbacks (loss of genome integrity) of TE proliferation. Notably, the expansion of the *AGO* gene family in *R. irregularis* appears to have been caused by TE activity, suggesting an ongoing AGO-TE co-evolution process in AM fungi (Dallaire *et al.*, 2021). TEs are also the primary source of sRNA production (49%), consistently with almost half of the genome constituted by repetitive elements. Interestingly, the remaining sRNA loci, corresponding to unannotated regions (41%) or protein-coding genes (10%), are preferentially localized near TEs, raising questions about the biological implications of such a linkage between non-TE and TE sRNA-generating loci. Remarkably, in the fungal pathogen *Botrytis cinerea* sRNAs, derived from TE were shown to move from fungus to plant cells and silence plant defense genes to favor host colonization (Weiberg *et al.*, 2013).

Although the most abundant DNA epigenetic change in AM fungi is 5-methylcytosine, it is worth noting that AM fungi present a unique methylome signature not present in Dikarya and plants: N6-methyldeoxyadenin at symmetrical ApT motifs (6mApT; Chaturvedi *et al.*, 2021). This feature preferentially occurs at gene and promoter regions and is associated with increased transcription. Core genes for symbiosis, like those involved in phosphate metabolism and transport, appear to be primarily 6mApT methylated.

Chromosome-level genome sequencing coupled to Hi-C analyses of *R. irregularis* strains showed that nuclei are organized in a euchromatic compartment, containing many genes with core functions, and a heterochromatic and heavily methylated compartment, rich in repetitive sequences but also in genes for predicted secreted proteins highly expressed *in planta* (Yildirir *et al.*, 2022; Sperschneider *et al.*, 2023). This suggests that during the symbiotic phase the chromatin condensation in AM fungi could be, to some extent, under the control of the host plant. On the contrary, there is also evidence that colonization by AM fungi causes changes in DNA methylation (Varga & Soulsbury, 2017, 2019) and induces the expression of specific retrotransposons in the host plant (Vangelisti *et al.*, 2019). Remarkably, the RiNEL1 effector from *R. irregularis* was recently shown to interact with the plant nucleosome protein histone 2B resulting in the downregulation of defense-related gene expression and enhanced mycorrhization (Wang *et al.*, 2021). Chromatin changes and TGS thus appear as another level of regulation of gene expression in the AM symbiosis, in which sRNAs could also play a role.

Considering the high number of sRNAs mapping on the genome of viruses hosted by AM fungi, the RNAi machinery of AM fungi is likely also involved in antiviral defense, although the role of these mycoviruses is still unknown (Silvestri *et al.*, 2020).

Is there cross-kingdom RNAi in the AM symbiosis?

The process where mobile sRNAs are transported between distantly related organisms leading to silencing the expression of target genes in the interacting partner through RNAi is known as cross-kingdom RNAi (ckRNAi, Cai *et al.*, 2018a) and has been described in plant and animal systems (Buck *et al.*, 2014; Cai *et al.*, 2019; Zhao *et al.*, 2021). In plants, numerous studies have confirmed

ckRNAi as a widespread defense strategy to suppress virulence targets in the invading organism (Zhang *et al.*, 2016; Cai *et al.*, 2018b; Hou *et al.*, 2019; Zhu *et al.*, 2022). Equally, evidences of sRNA movement from pathogens to plants, leading to the RNAi-mediated suppression of plant defense, pointed to ckRNAi as an evolutionarily conserved virulence mechanism (Weiberg *et al.*, 2013; Wang *et al.*, 2016; Xu *et al.*, 2022; Cheng *et al.*, 2023).

Building on the breadth of knowledge in plant pathosystems, the interest on ckRNAi has also moved toward mutualistic interactions (Qiao *et al.*, 2023): The first evidence for ckRNAi in such an interaction was described in the root nodule symbiosis where tRNA-derived sRNA fragments (tRFs) from *Bradyrhizobium japonicum* were found to hijack soybean AGO1 to target root development genes essential for nodule formation (Ren *et al.*, 2019). Similarly, tRFs from *Rhizobium tropici*, that target *Phaseolus vulgaris* genes also implicated in nodules, were shown to immuno-precipitate with the *P. vulgaris* AGO5 (Sánchez-Correa *et al.*, 2022). Convincing evidence for a role of ckRNAi during mycorrhizal symbioses came from a study on the ectomycorrhizal fungus *Pisolithus microcarpus* and its host plant *Eucalyptus grandis*. Fluorescence *in situ* hybridization experiments demonstrated the transfer of a fungal miRNA-like sequence (*Pmic_miR-8*) into root cells of the host plant where *Pmic_miR-8* was supposed to target a number of genes within the largest class of plant NLRs to sustain the interaction (Wong-Bajracharya *et al.*, 2022).

In AM symbiosis, there are indirect evidences in support of extracellular sRNA to modulate plant–fungal communication through ckRNAi. Following its discovery in plants, host-induced gene silencing (HIGS) emerged as a powerful genetic tool in crops to confer resistance to plant pathogens and pests, including fungi, oomycetes and insects (Zand Karimi & Innes, 2022). Similarly, in AM symbiosis HIGS and virus-induced gene silencing of RNAi (VIGS) emerged as useful tools to silence fungal genes in mycorrhizal roots and validate their relevance in the AM symbiosis. For example, a hairpin RNAi (hpRNAi)-silencing construct targeting the *R. irregularis Monosaccharide Transporter2* (*MST2*) expressed in *M. truncatula* hairy roots reduced *MST2* expression and mycorrhization levels (Helber *et al.*, 2011). HIGS/VIGS has since confirmed the function of a *R. irregularis* SL-induced secreted protein1 (RiSlS1; Tsuzuki *et al.*, 2016), a crinkler effector (RiCRN1; Voß *et al.*, 2018), a fungal aquaporin (Kikuchi *et al.*, 2016), a 14–3-3 (Ri14-3-3, Sun *et al.*, 2018), a lysin motif effector (Zeng *et al.*, 2020) and three HOG1-MAPK cascade proteins (RiSte11, RiPbs2 and RiHog1, Wang *et al.*, 2023), as well as a phosphate transporter (GigmPT) in the distantly related AM fungus, *Gigaspora margarita* (Xie *et al.*, 2016). Although evidence of naturally occurring extracellular sRNA transfer from plants to AM fungi is lacking, the success of HIGS/VIGS provided indirect confirmation of a ckRNAi mechanism in the AM symbiosis and confirmed that a RNAi-related mechanism to process host-derived sRNA exists in AM fungi.

Mining of sRNA sequences in *R. irregularis* showed an abundance of sRNA (*Rir*-sRNAs) highly expressed in mycorrhizal roots and presenting a characteristic 5' U enrichment, suggesting the potential for fungal sRNA to hijack the plant AGO1 RNAi machinery (Silvestri *et al.*, 2019, 2020; Dallaire *et al.*, 2021). Alongside, *in silico* sRNA-mRNA host target prediction provided a first indication of ckRNAi from AM fungi to their hosts. A recent study experimentally corroborated an *in silico* target prediction, showing that a sRNA from *R. irregularis* targets a *M. truncatula* WRKY transcription factor, controlling AM colonization level, suggesting the occurrence of fungus to plant ckRNAi (Silvestri *et al.*, 2023).

Little is known about the mechanisms that underpin ckRNAi, although in recent years, there has been an increase in studies that relate EVs in extracellular RNA delivery. EVs are defined as cell-derived nanoparticles delimited by a lipid bilayer that cannot replicate and that mediate the transport of proteins, lipids, RNAs and other biomolecules between cells and inter-organisms. EVs include entities such as apoptotic bodies, membrane-derived microvesicles and multi-vesicular body-derived exosomes (Théry *et al.*, 2018). A role for EVs in cross-kingdom transport of sRNAs was shown in landmark studies reporting that cells of Arabidopsis secreted exosome-like EVs containing sRNA cargoes that contributed to resistance against the filamentous pathogens *B. cinerea* (Cai *et al.*, 2018b) and *Phytophthora capsica* (Hou *et al.*, 2019). Moreover, a recent study showed that sRNAs from *B. cinerea* move through EVs to enter plant cells through clathrin-mediated endocytosis (He *et al.*, 2023). A similar role for EVs in transporting sRNAs in the AM symbiosis was proposed after transmission electron microscopy revealed a heterogenous collection of EVs, including exosome-like vesicles, accumulate in the apoplastic matrix between the host PAM and fungal arbuscule cell wall (Ivanov *et al.*, 2019; Roth *et al.*, 2019; Holland & Roth, 2023). Although such structural studies point to EVs as potential mediators of ckRNAi, future research is needed to confirm or refute EVs as shuttles for inter- and intra-organismal exRNA molecule transfer and to uncover possible mechanisms of sRNA loading and EV-mediated sRNA delivery in the context of ckRNAi in the AM symbiosis.

Open questions and future perspectives

sRNAs are emerging as core components of the complex regulation networks behind the cellular and metabolic changes that occur upon AM colonization. However, we are far from having a comprehensive picture as many issues remain to be elucidated (Fig. 2).

Investigations with computational, cellular, molecular and genetic tools should clarify the functional role of the plethora of plant sRNAs which are differentially expressed in the host plants during the AM symbiosis. Do they have a biological function at local (root) level, likely in the presymbiotic dialogue (Middleton *et al.*, 2023) or during fungal accommodation and the activation of metabolic pathways involved in nutrient exchanges? Are they mobile molecules that travel toward epigean organs and are involved in the systemic effect? Are there components of the RNAi machinery (i.e. AGO) specifically active/required in the AM symbiosis?

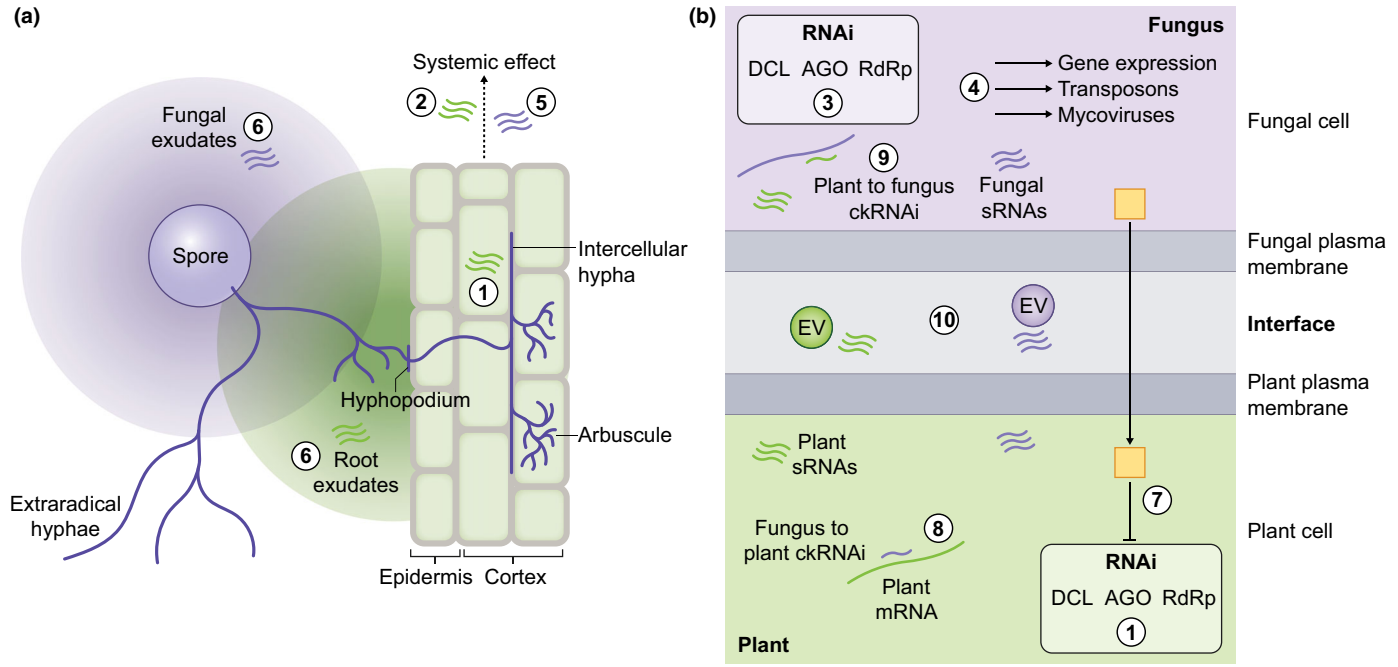

Fig. 2 Schematic representation of the open questions concerning small RNAs (sRNAs) in arbuscular mycorrhizal (AM) symbiosis, in particular referred to the plant (1–2), the fungus (3–5) and the plant–fungus interaction (6–10) (Panel a, b). (1) Which plant RNA interference (RNAi) components and sRNAs are involved in the AM symbiosis at root level? (2) Are there mobile plant sRNAs responsible of the systemic effect exerted by the AM symbiosis? (3) What is the function of the rich repertoire of ARGONAUTE (AGO) and RNA-dependent RNA polymerase (RdRp) in AM fungi? (4) What is the role of sRNAs in AM fungi and their impact on the fungal genome (endogenous genes and transposons) and on mycoviruses, and symbiotic functions? (5) Do fungal sRNAs travel to epigean organs? (6) Are sRNAs present in the plant/fungal exudates that are involved in the molecular dialogue between the partners at the presymbiotic stage? (7) Do AM fungi produce silencing suppressors controlling the plant RNAi machinery in analogy to plant pathogens? (8) Does fungus-to-plant cross-kingdom RNA interference (ckRNAi) occur? (9) Does plant to fungus ckRNAi occur? (10) Are extracellular vesicles (EVs) involved in the movement of small RNAs (sRNAs) from AM fungi to plant cells and vice versa? Panel (b) shows a magnification of the plant–fungus interaction that may involve different fungal structures (hyphopodia, intercellular hyphae and arbuscules).

In the arm race between viruses and plants, viruses were shown to produce silencing suppressors to fight against the host RNAi (Lopez-Gomollon & Baulcombe, 2022). Notably, effectors that suppress host RNA silencing have also been identified in bacterial (Navarro *et al.*, 2008), oomycete (Qiao *et al.*, 2013; Ye & Ma, 2016; Vetukuri *et al.*, 2017; Hou *et al.*, 2019) and fungal pathogens (Yin *et al.*, 2019; Zhu *et al.*, 2022). Since filamentous pathogens translocate silencing suppressors that disrupt sRNA production in plant host cells (Hou *et al.*, 2019), it is tempting to speculate that AM fungi could possess modulators of the host RNA silencing machinery to optimize the colonization process. A further level of complexity is given by the presence of mycoviruses in AM fungi, that may also be involved in the production of silencing suppressors. A recent study demonstrated that mycoviruses from an orchid mycorrhizal fungus encode for proteins that function as RNA silencing suppressors in fungal and in plant cells (Shimura *et al.*, 2022), suggesting a fascinating hypothesis that, using this strategy within a close association such as endomycorrhizas, viruses can be bidirectionally transmitted between plants and fungi.

On the fungal side, it will be crucial, though challenging due to their nature of obligate biotrophs, to functionally dissect the rich repertoire of AGO and RdRp to verify whether specific components are required for symbiosis in analogy to what is emerging from fungal pathogens (Cheng *et al.*, 2023). We also need to decipher the role of the sRNA populations in AM fungi to understand how they influence fungal gene expression and, as a consequence, fungal biology and symbiotic functions.

Considering the intimate nature of the interaction between plants and AM fungi, another challenge will be to understand whether silencing competent sRNAs are exchanged between the symbiotic partners of AM in both directions (Qiao *et al.*, 2023). The validation of *in silico* predicted targets of sRNA-mRNA pairs is a key goal to be achieved to provide convincing evidence on the presence of ckRNAi. And, if this is the case, we need to know how sRNAs move from one partner to the other. EVs are the first obvious candidate to be investigated; their characterization from mycorrhizal roots will shed light on molecules (sRNAs and proteins) that move between the two symbionts and their importance for mutualistic association.

Acknowledgements

LL is funded by the Project CN_00000033 under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4 – Call for tender no. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of the Italian Ministry of University and Research funded by the European Union – NextGenerationEU. The work in the MoRE laboratory is

funded by RTI2018-097262-B-I00 (funded by MCIN/AEI/10.13039/501100011033 and by 'ERDF A way of making Europe') and through the 'Severo Ochoa Programme for Centres of Excellence in R&D' 2016-2019 (SEV-2015-0533) and 2020-2023 (CEX2019-000902-S) funded by MCIN/AEI/10.13039/501100011033 and the CERCA program from the Generalitat de Catalunya. Postdoctoral fellowship to AS has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement N° 945043. RR is funded by the Royal Society Grant Code (URF\R1\201536).

Competing interests

None declared.

Author contributions

LL and IR-S conceptualized the manuscript. WCL, AS, VF, RR, IR-S and LL wrote the manuscript.

ORCID

Valentina Fiorilli https://orcid.org/0000-0001-9805-1559
Luisa Lanfranco https://orcid.org/0000-0002-3961-2552
William Conrad Ledford https://orcid.org/0000-0001-7130-2960
Ronelle Roth https://orcid.org/0000-0001-5566-8954
Ignacio Rubio-Somoza https://orcid.org/0000-0003-0754-816X
Alessandro Silvestri https://orcid.org/0000-0002-1635-7050

References

Bazin J, Khan GA, Combier JP, Bustos-Sanmamed P, Debernardi JM, Rodriguez R, Sorin C, Palatnik J, Hartmann C, Crespi M *et al.* 2013. miR396 affects mycorrhization and root meristem activity in the legume *Medicago truncatula*. *The Plant Journal* **74**: 920–934.

Bélanger S, Zhan J, Meyers C. 2023. Phylogenetic analyses of seven protein families refine the evolution of small RNA pathways in green plants. *Plant Physiology* **192**: 1183–1203.

Betti F, Ladera-Carmona MJ, Weits DA, Ferri G, Iacopino S, Novi G, Svezia B, Kunkowska AB, Santaniello A, Piaggesi A *et al.* 2021. Exogenous miRNAs induce post-transcriptional gene silencing in plants. *Nature Plants* **7**: 1379–1388.

Boccara M, Sarazin A, Thiébeauld O, Jay F, Voinnet O, Navarro L, Colot V. 2014. The Arabidopsis miR472-RDR6 silencing pathway modulates PAMP- and effector-triggered immunity through the post-transcriptional control of disease resistance genes. *PLoS Pathogens* **10**: e1003883.

Branscheid A, Sieh D, Pant BD, May P, Devers EA, Elkrog A, Krajinski F. 2010. Expression pattern suggests a role of miR399 in the regulation of the cellular response to local Pi increase during arbuscular mycorrhizal symbiosis. *Molecular Plant–Microbe Interactions* **23**: 915–926.

Bravo A, York T, Pumplin N, Mueller LA, Harrison MJ. 2016. Genes conserved for arbuscular mycorrhizal symbiosis identified through phylogenomics. *Nature Plants* **2**: 15208.

Buck A, Coakley G, Simbari F, McSorley HJ, Quintana JF, Le Bihan T, Kumar S, Abreu-Goodger C, Lear M, Harcus Y *et al.* 2014. Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity. *Nature Communications* **5**: 5488.

Cai Q, He B, Kogel KH, Jin H. 2018a. Cross-kingdom RNA trafficking and environmental RNAi-nature's blueprint for modern crop protection strategies. *Current Opinion in Microbiology* **46**: 58–64.

Cai Q, He B, Weiberg A, Buck AH, Jin H. 2019. Small RNAs and extracellular vesicles: new mechanisms of cross-species communication and innovative tools for disease control. *PLoS Pathogens* **15**: e1008090.

Cai Q, Qiao L, Wang M, He B, Lin FM, Palmquist J, Meyers BC. 2018b. Plants send small RNAs in extracellular vesicles to fungal pathogen to silence virulence genes. *Science* **360**: 1126–1129.

Cenci A, Rouard M. 2017. Evolutionary analyses of GRAS transcription factors in angiosperms. *Frontiers in Plant Science* **8**: 273.

Chang SS, Zhang Z, Liu Y. 2012. RNA interference pathways in fungi: mechanisms and functions. *Annual Review of Microbiology* **66**: 305–323.

Chaturvedi A, Cruz Corella J, Robbins C, Loha A, Menin L, Gasilova N, Masclaux FG, Lee SJ, Sanders IR. 2021. The methylome of the model arbuscular mycorrhizal fungus, *Rhizophagus irregularis*, shares characteristics with early diverging fungi and Dikarya. *Communications Biology* **4**: 901.

Cheng A, Lederer B, Oberkofler L, Huang L, Platten F, Dunker F, Tisserant C, Weiberg A. 2023. A fungal RNA-dependent RNA polymerase is a novel player in plant infection and cross-kingdom RNA interference. *bioRxiv*. doi: 10.1101/2023.06.02.543307.

Chialva M, Patono DL, Perez de Souza L, Novero M, Vercellino S, Maghrebi M, Morgante M, Lovisolo C, Vigani V, Fernie A *et al.* 2023. The mycorrhizal root-shoot axis elicits *Coffea arabica* growth under low phosphate conditions. *New Phytologist* **239**: 271–285.

Couzigou JM, Lauressergues D, Andre O, Gutjahr C, Guillotin B, Bécard G, Combier JP. 2017. Positive gene regulation by a natural protective miRNA enables arbuscular mycorrhizal symbiosis. *Cell Reports* **20**: 1339–1350.

Dallaire A, Manley BF, Wilkens M, Bista I, Quan C, Evangelisti E, Bradshaw CR, Ramakrishna NB, Schornack S, Butter F *et al.* 2021. Transcriptional activity and epigenetic regulation of transposable elements in the symbiotic fungus *Rhizophagus irregularis*. *Genome Research* **31**: 2290–2302.

Das D, Paries M, Hobecker K, Gigl M, Dawid C, Lam H, Zhang J, Chen M, Gutjahr C. 2022. Phosphate starvation response transcription factors enable arbuscular mycorrhiza symbiosis. *Nature Communications* **13**: 477.

Delaux PM, Varala K, Edger PP, Coruzzi GM, Pires JC, Ané JM. 2014. Comparative phylogenomics uncovers the impact of microbial symbionts on host genome evolution. *PLoS Genetics* **10**: e1004487.

Devers EA, Branscheid A, May P, Krajinski F, Bäumlein H. 2011. Stars and symbiosis: microRNA- and microRNA*-mediated transcript cleavage involved in arbuscular mycorrhizal symbiosis. *Plant Physiology* **156**: 1990–2010.

Etemadi M, Gutjahr C, Couzigou JM, Zouine M, Lauressergues D, Timmers A, Audran C, Bouzayen M, Bécard G, Combier JP. 2014. Auxin perception is required for arbuscule development in arbuscular mycorrhizal symbiosis. *Plant Physiology* **166**: 281–292.

Fiorilli V, Vannini C, Ortolani F, Garcia-Seco D, Chiapello M, Novero M, Domingo G, Terzi V, Morcia C, Bagnaresi P *et al.* 2018. Omics approaches revealed how arbuscular mycorrhizal symbiosis enhances yield and resistance to leaf pathogen in wheat. *Scientific Reports* **8**: 9625.

Formey D, Sallet E, Lelandais-Brière C, Ben C, Bustos-Sanmamed P, Niebel A, Frugier F, Combier JP, Debellé F, Hartmann C *et al.* 2014. The small RNA diversity from *Medicago truncatula* roots under biotic interactions evidences the environmental plasticity of the miRNAome. *Genome Biology* **15**: 457.

Genre A, Lanfranco L, Perotto S, Bonfante P. 2020. Unique and common traits in mycorrhizal symbioses. *Nature Reviews Microbiology* **18**: 649–660.

Gobbato E, Marsh JF, Vernié T, Wang E, Maillet F, Kim J, Miller JB, Sun J, Bano SA, Ratet P *et al.* 2012. A GRAS-type transcription factor with a specific function in mycorrhizal signaling. *Current Biology* **22**: 2236–2241.

Gu M, Xu K, Chen A, Zhu Y, Tang G, Xu G. 2010. Expression analysis suggests potential roles of microRNAs for phosphate and arbuscular mycorrhizal signaling in *Solanum lycopersicum*. *Physiologia Plantarum* **138**: 226–237.

He B, Hamby R, Jin H. 2021. Plant extracellular vesicles: Trojan horses of cross-kingdom warfare. *FASEB Bioadvances* **3**: 657–664.

He B, Wang H, Liu G, Chen A, Cai Q, Jin H. 2023. Fungal small RNAs ride in extracellular vesicles to enter plant cells through clathrin-mediated endocytosis. *Nature Communications* **14**: 4383.

Helber N, Wippel K, Sauer N, Schaarschmidt S, Hause B, Requena N. 2011. A versatile monosaccharide transporter that operates in the arbuscular mycorrhizal fungus *Glomus* sp. is crucial for the symbiotic relationship with plants. *Plant Cell* **23**: 3812–3823.

Hobecker KV, Reynoso MA, Bustos-Sanmamed P, Wen J, Mysore KS, Crespi M, Blanco FA, Zanetti ME. 2017. The microRNA390/TAS3 pathway mediates symbiotic nodulation and lateral root growth. *Plant Physiology* **174**: 2469–2486.

Hofferek V, Mendrinna A, Gaude N, Krajinski F, Devers EA. 2014. MiR171h restricts root symbioses and shows like its target NSP2 a complex transcriptional regulation in *Medicago truncatula*. *BMC Plant Biology* **14**: 199.

Holland S, Roth R. 2023. Extracellular vesicles in the arbuscular mycorrhizal symbiosis: current understanding and future perspectives. *Molecular Plant–Microbe Interactions* **36**: 235–244.

Hou Y, Zhai Y, Feng L, Karimi HZ, Rutter BD, Zeng L, Choi DS, Zhang B, Gu W, Chen X *et al.* 2019. A Phytophthora effector suppresses trans-kingdom RNAi to promote disease susceptibility. *Cell Host & Microbe* **25**: 153–165.

Huang C-Y, Wang H, Hu P, Hamby R, Jin H. 2019. Small RNAs – big players in plant–microbe interactions. *Cell Host & Microbe* **26**: 173–182.

Ivanov S, Austin J, Berg RH, Harrison MJ. 2019. Extensive membrane systems at the host–arbuscular mycorrhizal fungus interface. *Nature Plants* **5**: 194–203.

Ivanov S, Harrison MJ. 2018. Accumulation of phosphoinositides in distinct regions of the periarbuscular membrane. *New Phytologist* **221**: 2213–2227.

Kaushal R, Peng L, Singh SK, Zhang M, Zhang X, Vílchez J, Wang Z, He D, Yang Y *et al.* 2021. Dicer-like proteins influence Arabidopsis root microbiota independent of RNA-directed DNA methylation. *Microbiome* **9**: 57.

Kikuchi Y, Hijikata N, Ohtomo R, Handa Y, Kawaguchi M, Saito K, Masuta C, Ezawa T. 2016. Aquaporin-mediated long-distance polyphosphate translocation directed towards the host in arbuscular mycorrhizal symbiosis: application of virus-induced gene silencing. *New Phytologist* **211**: 1202–1208.

Lanfranco L, Bonfante P. 2023. Lessons from arbuscular mycorrhizal fungal genomes. *Current Opinion in Microbiology* **75**: 102357.

Lauressergues D, Delaux PM, Formey D, Lelandais-Brière C, Fort S, Cottaz S, Bécard G, Niebel A, Roux C, Combier JP. 2012. The microRNA miR171h modulates arbuscular mycorrhizal colonization of *Medicago truncatula* by targeting NSP2. *The Plant Journal* **72**: 512–522.

Lax C, Tahiri G, Patiño-Medina JA, Cánovas-Márquez JT, Pérez-Ruiz JA, Osorio-Concepción M, Navarro E, Calo S. 2020. The evolutionary significance of RNAi in the fungal kingdom. *International Journal of Molecular Sciences* **21**: 9348.

Lee S, Kong M, Harrison P, Hijri M. 2018. Conserved proteins of the RNA Interference system in the arbuscular mycorrhizal fungus *Rhizoglomus irregulare* provide new insight into the evolutionary history of Glomeromycota. *Genome Biology and Evolution* **10**: 328–343.

Li S, Wang X, Xu W, Liu T, Cai C, Chen L, Clark CB, Ma J. 2021. Unidirectional movement of small RNAs from shoots to roots in interspecific heterografts. *Nature Plants* **7**: 50–59.

Li Y, Kim EJ, Voshall A, Moriyama EN, Cerutti H. 2023. Small RNAs >26 nt in length associate with AGO1 and are upregulated by nutrient deprivation in the alga Chlamydomonas. *Plant Cell* **35**: 1868–1887.

Li Y, Zhang Q, Zhang J, Wu L, Qi Y, Zhou JM. 2010. Identification of microRNAs involved in pathogen-associated molecular pattern-triggered plant innate immunity. *Plant Physiology* **152**: 2222–2231.

Li Z, Li W, Guo M, Liu S, Liu L, Yu Y, Mo B, Chen X, Gao L. 2022. Origin, evolution and diversification of plant ARGONAUTE proteins. *The Plant Journal* **109**: 1086–1097.

Lopez-Gomollon S, Baulcombe DC. 2022. Roles of RNA silencing in viral and non-viral plant immunity and in the crosstalk between disease resistance systems. *Nature Reviews Molecular Cell Biology* **23**: 645–662.

López-Márquez D, Del-Espino Á, López-Pagán N, Rodríguez-Negrete EA, Rubio-Somoza I, Ruiz-Albert J, Bejarano ER, Beuzón CR. 2021. miR825-5p targets the TIR-NBS-LRR gene MIST1 and down-regulates basal immunity against *Pseudomonas syringae* in Arabidopsis. *Journal of Experimental Botany* **72**: 7316–7334.

MacLean AM, Bravo A, Harrison MJ. 2017. Plant signaling and metabolic pathways enabling arbuscular mycorrhizal symbiosis. *Plant Cell* **10**: 2319–2335.

Manley BF, Lotharukpong JS, Barrera-Redondo J, Llewellyn T, Yildirir G, Sperschneider J, Corradi N, Paszkowski U, Miska EA, Dallaire A. 2023. A highly contiguous genome assembly reveals sources of genomic novelty in the symbiotic fungus *Rhizophagus irregularis*. *G3: Genes, Genomes, Genetics* **13**: jkad077.

Mendoza-Soto AB, Sánchez-López R, Gómez-Ariza J, Valdés-López O. 2022. Mycorrhizal colonization induces systemic changes in miRNA expression and targets in tomato leaves. *Plant Science* **317**: 111080.

Mewalal R, Yin H, Hu R, Jawdy SS, Gunter LE, Engle N, Tschaplinski TJ. 2019. Identification of populus small RNAs responsive to mutualistic interactions with mycorrhizal fungi, *Laccaria bicolor* and *Rhizophagus irregularis*. *Frontiers in Plant Science* **10**: 372.

Mi S, Cai T, Hu Y, Chen Y, Hodges E, Ni F, Jin H. 2008. Sorting of small RNAs into Arabidopsis argonaute complexes is directed by the 5′ terminal nucleotide. *Cell* **133**: 116–127.

Middleton H, Monard C, Daburon V, Clostres E, Tremblay J, Yergeau É, Abdelhak El Amrani A. 2023. Plants release miRNAs in the rhizosphere, targeting microbial genes. *bioRxiv*. doi: 10.1101/2022.07.26.501597.

Müller LM, Harrison MJ. 2019. Phytohormones, miRNAs, and peptide signals integrate plant phosphorus status with arbuscular mycorrhizal symbiosis. *Current Opinion in Plant Biology* **50**: 132–139.

Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JDG. 2006. A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* **312**: 436–439.

Navarro L, Jay F, Nomura K, He SY, Voinnet O. 2008. Suppression of the microRNA pathway by bacterial effector proteins. *Science* **321**: 964–967.

Oldroyd GED. 2013. Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nature Reviews Microbiology* **11**: 252–263.

Olesen MT, Kristensen SL. 2021. Circular RNAs as microRNA sponges: evidence and controversies. *Essays in Biochemistry* **65**: 685–696.

Ono S, Liu H, Tsuda K, Fukai E, Tanaka K, Sasaki T, Nonomura KI. 2018. EAT1 transcription factor, a non-cell-autonomous regulator of pollen production, activates meiotic small RNA biogenesis in rice anther tapetum. *PLoS Genetics* **14**: e1007238.

Pandey P, Wang M, Baldwin IT, Pandey SP, Groten K. 2018. Complex regulation of microRNAs in roots of competitively-grown isogenic *Nicotiana attenuata* plants with different capacities to interact with arbuscular mycorrhizal fungi. *BMC Genomics* **19**: 937.

Pérez-Arques C, Navarro-Mendoza MI, Murcia L, Navarro E, Garre V, Nicolás FE. 2020. A non-canonical RNAi pathway controls virulence and genome stability in Mucorales. *PLoS Genetics* **16**: e1008611.

Pimprikar P, Gutjahr C. 2018. Transcriptional regulation of arbuscular mycorrhiza development. *Plant Cell Physiology* **59**: 673–690.

Pradhan M, Baldwin IT, Pandey SP. 2023. Argonaute7 (AGO7) optimizes arbuscular mycorrhizal fungal associations and enhances competitive growth in *Nicotiana attenuata*. *New Phytologist* **240**: 382–398.

Pradhan S, Requena N. 2022. Distinguishing friends from foes: can smRNAs modulate plant interactions with beneficial and pathogenic organisms? *Current Opinion in Plant Biology* **64**: 102259.

Qiao SA, Gao Z, Roth R. 2023. A perspective on cross-kingdom RNA interference in mutualistic symbioses. *New Phytologist* **240**: 68–79.

Qiao Y, Liu L, Xiong Q, Flores C, Wong J, Shi J, Wang X, Liu X, Xiang Q, Jiang S *et al.* 2013. Oomycete pathogens encode RNA silencing suppressors. *Nature Genetics* **45**: 330–333.

Ren B, Wang X, Duan J, Ma J. 2019. Rhizobial tRNA-derived small RNAs are signal molecules regulating plant nodulation. *Science* **365**: 919–922.

Rich MK, Courty PE, Roux C, Reinhardt D. 2017. Role of the GRAS transcription factor ATA/RAM1 in the transcriptional reprogramming of arbuscular mycorrhiza in *Petunia hybrida*. *BMC Genomics* **18**: 589.

Roth R, Chiapello M, Montero H, Gehrig P, Grossmann J, O'Holleran K, Hartken D, Walters F, Yang SY, Stefan Hillmer S *et al.* 2018. A rice serine/threonine receptor-like kinase regulates arbuscular mycorrhizal symbiosis at the peri-arbuscular membrane. *Nature Communications* **9**: 4677.

Roth R, Hillmer S, Funaya C, Chiapello M, Schumacher K, Lo Presti L, Kahmann R, Paszkowski U. 2019. Arbuscular cell invasion coincides with extracellular vesicles and membrane tubules. *Nature Plants* **5**: 204–211.

Sánchez-Correa MS, Isidra-Arellano MC, Pozas-Rodríguez EA, Reyero-Saavedra MR, Morales-Salazar A, del Castillo SML-C, Sánchez-Flores A, Jiménez Jacinto V, Reyes JL, Formey D *et al.* 2022. Argonaute5 and its associated small RNAs

modulate the transcriptional response during the rhizobia-*Phaseolus vulgaris* symbiosis. *Frontiers in Plant Science* **13**: 1034419.

Sanobar N, Lin PC, Pan ZJ, Fang RY, Tjita V, Chen FF, Wang HC, Tsai HL, Wu SH, Shen TL *et al.* 2021. Investigating the viral suppressor HC-Pro inhibiting small RNA methylation through functional comparison of HEN1 in Angiosperm and Bryophyte. *Viruses* **13**: 1837.

Shahid S, Kim G, Johnson NR, Wafula E, Wang F, Coruh C, Timko MP. 2018. MicroRNAs from the parasitic plant *Cuscuta campestris* target host messenger RNAs. *Nature* **553**: 82–85.

Shi J, Zhao B, Zheng S, Zhang X, Wang X, Dong W, Xie Q, Wang G, Xiao Y, Chen F *et al.* 2021. A phosphate starvation response-centered network regulates mycorrhizal symbiosis. *Cell* **184**: 5527–5540.

Shimura H, Kim H, Matsuzawa A, Akino S, Masuta C. 2022. Coat protein of partitiviruses isolated from mycorrhizal fungi functions as an RNA silencing suppressor in plants and fungi. *Scientific Reports* **12**: 7855.

Silvestri A, Fiorilli V, Miozzi L, Accotto GP, Turina M, Lanfranco L. 2019. *In silico* analysis of fungal small RNA accumulation reveals putative plant mRNA targets in the symbiosis between an arbuscular mycorrhizal fungus and its host plant. *BMC Genomics* **20**: 169.

Silvestri A, Ledford W, Fiorilli V, Votta C, Scerna A, Tucconi J, Mocchetti A, Grasso G, Balestrini R, Jin H *et al.* 2023. Cross-kingdom RNAi adds a new layer of communication to the arbuscular mycorrhizal symbiosis. doi: 10.21203/rs.3.rs-3423345/v1.

Silvestri A, Turina M, Fiorilli V, Miozzi L, Venice F, Bonfante P, Lanfranco L. 2020. Different genetic sources contribute to the small RNA population in the arbuscular mycorrhizal fungus *Gigaspora margarita*. *Frontiers in Microbiology* **11**: 395.

Smith NA, Eamens AL, Wang MB. 2011. Viral small interfering RNAs target host genes to mediate disease symptoms in plants. *PLoS Pathogens* **7**: e1002022.

Soto-Suárez M, Baldrich P, Weigel D, Rubio-Somoza I, San SB. 2017. The Arabidopsis miR396 mediates pathogen-associated molecular pattern-triggered immune responses against fungal pathogens. *Scientific Reports* **7**: 44898.

Sperschneider J, Yildirir G, Rizzi Y, Malar M, Sorwar E, Chen ECH, Iwasaki W, Brauer EK, Bosnich W, Gutjahr C *et al.* 2023. Arbuscular mycorrhizal fungi heterokayons have two nuclear populations with distinct roles in host plant interactions. *Nature Microbiology* **8**:2142–2453.

Sun Z, Song J, Xin X, Xie X, Zhao B. 2018. Arbuscular mycorrhizal fungal 14-3-3 proteins are involved in arbuscule formation and responses to abiotic stresses during AM symbiosis. *Frontiers in Microbiology* **9**: 91.

Takeda A, Iwasaki S, Watanabe Y. 2008. The mechanism selecting the guide strand from small RNA duplexes is different among Argonaute proteins. *Plant Cell Physiology* **49**: 493–500.

Teng C, Zhang H, Hammond R, Huang K, Meyers BC, Walbot V. 2020. Dicer-like 5 deficiency confers temperature-sensitive male sterility in maize. *Nature Communications* **11**: 2912.

Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A, Arab T, Archer F, Atkin-Smith GK *et al.* 2018. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of Extracellular Vesicles* **7**: 1535750.

Torres-Martínez S, Ruiz-Vázquez RM. 2017. RNAi in fungi: a varied landscape of small RNAs and biological functions. *Annual Review of Microbiology* **71**: 371–391.

Trieu TA, Calo S, Nicolás FE, Vila A, Moxon S, Dalmay T, Ruiz-Vázquez RM. 2015. A non-canonical RNA silencing pathway promotes mRNA degradation in basal fungi. *PLoS Genetics* **11**: e1005168.

Tsuzuki S, Handa Y, Takeda N, Kawaguchi M. 2016. Strigolactone-induced putative secreted protein 1 is required for the establishment of symbiosis by the arbuscular mycorrhizal fungus *Rhizophagus irregularis*. *Molecular Plant–Microbe Interactions* **4**: 277–286.

Vangelisti A, Mascagni F, Giordani T, Sbrana C, Turrini A, Cavallini A, Giovannetti M, Natali L. 2019. Arbuscular mycorrhizal fungi induce the expression of specific retrotransposons in roots of sunflower (*Helianthus annuus* L.). *PLoS ONE* **14**: e0212371.

Varga S, Soulsbury CD. 2017. Paternal arbuscular mycorrhizal fungal status affects DNA methylation in seeds. *Biology Letters* **13**: 20170407.

Varga S, Soulsbury CD. 2019. Arbuscular mycorrhizal fungi change host plant DNA methylation systemically. *Plant Biology* **21**: 278–283.

Vasseur F, Baldrich P, Jiménez-Góngora T, Villar Martin L, Weigel D, Rubio-Somoza I. 2022. miR472 deficiency enhances *Arabidopsis thaliana* defence without reducing seed production. *bioRxiv*. doi: 10.1101/2022.12.13.520224.

Vetukuri RR, Whisson SC, Grenville-Briggs LJ. 2017. *Phytophthora infestans* effector Pi14054 is a novel candidate suppressor of host silencing mechanisms. *European Journal of Plant Pathology* **149**: 771–777.

Volpe V, Chialva M, Mazzarella T, Crosino A, Capitanio S, Costamagna L, Kohlen W, Genre A. 2023. Long-lasting impact of chitooligosaccharide application on strigolactone biosynthesis and fungal accommodation promotes arbuscular mycorrhiza in *Medicago truncatula*. *New Phytologist* **236**: 2316–2331.

Voß S, Betz R, Heidt S, Corradi N, Requena N. 2018. RiCRN1, a crinkler effector from the arbuscular mycorrhizal fungus *Rhizophagus irregularis*, functions in arbuscule development. *Frontiers in Microbiology* **9**: 2068.

Wang M, Weiberg A, Lin FM, Thomma B, Huang HD, Jin H. 2016. Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nature Plants* **2**: 16151.

Wang P, Jiang H, Boeren S, Dings H, Kulikova O, Bisseling T, Limpens E. 2021. A nuclear-targeted effector of *Rhizophagus irregularis* interferes with histone 2B mono-ubiquitination to promote arbuscular mycorrhisation. *New Phytologist* **230**: 1142–1155.

Wang S, Xie X, Che X, Lai W, Ren Y, Fan X, Hu W, Tang M, Chen H. 2023. Host- and virus-induced gene silencing of HOG1-MAPK cascade genes in *Rhizophagus irregularis* inhibit arbuscule development and reduce resistance of plants to drought stress. *Plant Biotechnology Journal* **4**: 866–883.

Weiberg A, Wang M, Lin FM, Zhao H, Zhang Z, Kaloshian I, Huang HD, Jin H. 2013. Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. *Science* **342**: 118–123.

Wong-Bajracharya J, Singan VR, Monti R, Plett KL, Ng V, Grigoriev IV, Martin FM, Anderson IC, Plett JM. 2022. The ectomycorrhizal fungus *Pisolithus microcarpus* encodes a microRNA involved in cross-kingdom gene silencing during symbiosis. *Proceedings of the National Academy of Sciences, USA* **119**: e2103527119.

Wu P, Wu Y, Liu CC, Liu LW, Ma FF, Wu XY, Wu M, Hang YY, Chen JQ, Shao ZQ *et al.* 2016. Identification of arbuscular mycorrhiza (AM)-responsive microRNAs in tomato. *Frontiers in Plant Science* **7**: 429.

Xie X, Lin H, Peng X, Xu C, Sun Z, Jiang K, Huang A, Wu X, Tang N, Salvioli A *et al.* 2016. Arbuscular mycorrhizal symbiosis requires a phosphate transceptor in the *Gigaspora margarita* fungal symbiont. *Molecular Plant* **9**: 1583–1608.

Xu M, Li G, Guo Y, Gao Y, Zhu L, Liu Z, Tian R, Gao C, Han P, Wang N *et al.* 2022. A fungal microRNA-like RNA subverts host immunity and facilitates pathogen infection by silencing two host receptor-like kinase genes. *New Phytologist* **6**: 2503–2519.

Xu Y, Zhu S, Liu F, Wang W, Wang X, Han G, Cheng B. 2018. Identification of arbuscular mycorrhiza fungi responsive microRNAs and their regulatory network in maize. *International Journal of Molecular Sciences* **19**: 3201.

Xue L, Cui H, Buer B, Vijayakumar V, Delaux PM, Junkermann S, Bucher M. 2015. Network of GRAS transcription factors involved in the control of arbuscule development in *Lotus japonicus*. *Plant Physiology* **167**: 854–871.

Ye W, Ma Z. 2016. The effector AVR3a of *Phytophthora infestans* manipulates RNA silencing to facilitate infection. *The Plant Journal* **83**: 669–679.

Yildirir G, Sperschneider J, Malar CM, Chen ECH, Iwasaki W, Cornell C, Corradi N. 2022. Long reads and Hi-C sequencing illuminate the two-compartment genome of the model arbuscular mycorrhizal symbiont *Rhizophagus irregularis*. *New Phytologist* **233**: 1097–1107.

Yin C, Ramachandran SR, Zhai Y, Bu C, Pappu HR, Hulbert SH. 2019. A novel fungal effector from *Puccinia graminis* suppressing RNA silencing and plant defense responses. *New Phytologist* **222**: 1566–1577.

Yu B, Yang Z, Li J, Minakhina S, Yang M, Padgett RW, Steward R. 2005. Methylation as a crucial step in plant microRNA biogenesis. *Science* **307**: 932–935.

Zand Karimi H, Baldrich P, Rutter B, Borniego L, Zajt K, Meyers B, Innes R. 2022. Arabidopsis apoplastic fluid contains sRNA- and circular RNA–protein complexes that are located outside extracellular vesicles. *Plant Cell* **34**: 1863–1881.

Zand Karimi H, Innes RW. 2022. Molecular mechanisms underlying host-induced gene silencing. *Plant Cell* **34**: 3183–3199.

Zeng T, Rodriguez-Moreno L, Mansurkhodzaev A, Wang P, van den Berg W, Gasciolli V, Cottaz S, Fort S, Thomma BPHJ, Bono JJ *et al.* 2020. A lysin motif effector subverts chitin- triggered immunity to facilitate arbuscular mycorrhizal symbiosis. *New Phytologist* **225**: 448–460.

Zeng Z, Liu Y, Feng XY, Li SX, Jiang XM, Chen JQ, Shao ZQ. 2023. The RNAome landscape of tomato during arbuscular mycorrhizal symbiosis reveals an evolving RNA layer symbiotic regulatory network. *Plant Communications* **4**: 100429.

Zhang T, Zhao YL, Zhao JH, Wang S, Jin Y, Chen ZQ, Fang YY, Hua CL, Ding SW, Guo HS. 2016. Cotton plants export microRNAs to inhibit virulence gene expression in a fungal pathogen. *Nature Plants* **2**: 16153.

Zhao JH, Zhang T, Liu QY, Guo HS. 2021. Trans-kingdom RNAs and their fates in recipient cells: advances, utilization, and perspectives. *Plant Communications* **2**: 100167.

Zhu C, Liu JH, Zhao JH, Liu T, Chen YY, Wang CH, Zhang ZH, Guo HS, Duan CG. 2022. A fungal effector suppresses the nuclear export of AGO1-miRNA complex to promote infection in plants. *Proceedings of the National Academy of Sciences, USA* **119**: e2114583119.