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Distinguishing friends from foes: Can smRNAs modulate plant interactions with beneficial and pathogenic organisms?

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Abstract
In their agro-ecological habitats, plants are constantly challenged by fungal interactions that might be pathogenic or beneficial in nature, and thus, plants need to exhibit appropriate responses to discriminate between them. Such interactions involve sophisticated molecular mechanism of signal exchange, signal transduction and regulation of gene expression. Small RNAs (smRNAs), including the microRNAs (miRNAs), form an essential layer of regulation in plant developmental processes as well as in plant adaptation to environmental stresses, being key for the outcome during plant–microbial interactions. Further, smRNAs are mobile signals that can go across kingdoms from one interacting partner to the other and hence can be used as communication as well as regulatory tools not only by the host plant but also by the colonising fungus. Here, largely with a focus on plant–fungal interactions and miRNAs, we will discuss the role of smRNAs, and how they might help plants to discriminate between friends and foes.

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smRNAs, Plant-fungal interactions, Pathogens, Mutualistic symbionts, Cross-kingdom RNAi, Argonautes.

Introduction

Non-coding small-RNAs (smRNAs) form an indispensable layer of gene expression regulation [1,2]. A large spectrum of endogenous smRNA, including the microRNAs (miRNAs) exists in plants, usually ranging between 18 and 30 nucleotides, and capable of inducing RNA interference [1,3–6]. smRNAs may act locally in the cells where they are produced or in a non-cell autonomous manner [7–10]. But most remarkably, they can be even translocated in a cross-kingdom manner between plants and their interacting organisms [6,11–15]. smRNAs regulate gene expression in a sequence dependent manner, impacting a broad range of plant processes beyond plant development to adapt to the environment including the interaction with other organisms [16–19].

In their agro-ecological habitats, plants are constantly challenged with fungal interactions that might have pathogenic as well as symbiotic outcomes. Such fungal encounters of host plants entail a large-scale reprograming of the smRNome, with examples both in pathogenic and symbiotic interactions [20–27]. Most interestingly, interactions with plants also modify the smRNome of the fungal partner [28–30].

What could be the consequences of reprograming of smRNAs/miRNAs for the interaction between plant and fungi is a key question. It is plausible that smRNAs serve as signal themselves. Then, smRNAs would not only be molecules that adjust plant response to the microbe, but also communication tools for the hosts to discriminate friends from foes and effective small weapons to control interacting fungi in a cross-kingdom manner. We shall examine such possibilities in the following sections.

Phytohormones: A hot spot for smRNA regulation during fungal interactions

Host responses to microbial infection are a consequence of a complex signal transduction process in which phytohormones such as jasmonic acid (JA) and its conjugates, salicylic acid (SA) and ethylene play a major role. While these defense hormones might have mutually exclusive roles, they are known to crosstalk in a synergistic as well as in an antagonistic manner [31,32]. Interactions between these traditional ‘defense’ hormones and other plant hormones further fine-tune plant microbial interactions [33–35]. Consistently, phytohormones also have been disclosed as having a major role in shaping the symbiosis with AM fungi, reviewed in the

Box 1. Hormonal crosstalk by miRNAs during plant–fungal interactions.

Both symbiotic and pathogenic fungi have been shown to modulate the auxin response in plants by means of several miRNAs. Thus, in many plants, including, tomato, barrel clover, rice, coyote tobacco and poplar, the precursor of miR393 was found to be downregulated during symbiosis with AM fungi [22,74,75]. miR393 targets, among others, the auxin receptors TIR1/AFB [76] and overexpression of miR393 led to a severe impairment of the development of the symbiotic fungus in the root [75]. miR393 seems to be a conserved hub where many plant-interacting organisms might impact on, not only fungi, but also bacteria or aphids, to control auxin-mediated defense responses [77]. It has been proposed that by negatively targeting the auxin receptors, miR393 increases the SA signalling and thus plants are more resistant to biotrophs but more susceptible to necrotrophs [44,77,78].

The auxin node is controlled, in addition, by several other miRNAs, for instance miR160 and miR167, that also target ARFs [79]. Interestingly, miR160, which is induced in response to infection by the oomycete *Phytophthora infestans* has been also proposed to mediate the antagonistic crosstalk between SA-related defences and auxin-regulated growth pathways during infection in potato [80]. miR160 and miR167 are both regulated during symbiosis with the AM fungus *R. irregularis* in *Medicago truncatula* [45]. New miRNAs targeting auxin signalling were identified in eggplants in response to infection with the pathogenic fungus *Verticillium dahliae* [81]. Interestingly, preliminary functional results suggest that induction of miRm0002 enhances resistance to *V. dahliae* not by cleaving its putative target ARF8 but by inhibiting its translation [82]. However, this possibility awaits confirmation.

Several other hormonal nodes where miRNAs have been shown to impact during plant–fungal interactions include ethylene response factors. For example can-miRn37a, a novel miRNA from chilli, can target ethylene response factors and act against infection of *Colletotrichum truncatum* [83]; miR163, a miRNA unique to the *Arabidopsis* genus, that invokes a regulatory module involving JA and SA signalling [84]; miR773 from *Arabidopsis,* that participates in the resistance against a variety of fungal pathogens (such as the necrotroph *Plectosphaerrella cucumerina*, or the hemibiotrophs *Fusarium oxysporum* and *Collettotrichum higginianum*) impacting on the JA, ethylene and SA-signalling pathways [85]; or the lily miR159, which overexpression represses two MYB transcription factor boosting both JA and SA pathway during infection of grey mould *Botrytis cinerea* [86]. How these other pathways are affected by miRNA during interaction with symbiotic fungi has not been functionally analysed yet, but there are several findings that suggest that ABA and ethylene pathways are also targeted [19,22,45].

study by Gutjahr et al. [36]. Phytohormones can alter the miRNome [37–39] and not surprisingly, smRNAs are also important factors regulating phytohormone crosstalks during plant–fungal interactions [20,22]. It is predicted, that sophisticated miRNA-transcription factor interactomes could fine-tune phytohormonal signalling during these interactions. For instance, WRKY transcription factors, which are induced by pathogens and by phytohormones, regulate smRNA populations [40]. But also, WRKY transcription factors can be themselves targets of pathogen-induced miRNAs, as shown in the interaction between *Magnaporthe oryzae* and rice [41]. The many ways phytohormonal pathways are intersected by miRNA during fungal–plant interactions are shown in Box 1.

Endogenous miRNAs generated in response to the interacting microbe are a blueprint that serves plant cell reprogramming

miRNAs networks may act in a complementary, synergistic or antagonistic fashion to help plants to maximise the appropriate physiological response. miRNAs and their targets allow a context-dependent regulation to tailor specificity in responding to foes and friends. Hence, the expression of miRNAs has been shown to change specifically in a stimulus dependent manner. Many of these miRNA networks act upstream regulating hormone biogenesis and their master transcription factors. *Fusarium brachygibbosum*-induced miRNAs, for instance, target JA biogenesis and many other JA signalling genes, largely affecting the functioning of the JA pathway [20]. However, although JA-signalling forms a central component of the defense against insect herbivores [42], the herbivore-induced miRNome in *Nicotiana attenuata* hardly overlaps with the one elicited by *F. brachygibbosum* [20]. Similarly, *N. attenuata* miRNAs regulated in response to *Rhizophagus irregularis* colonisation [22] revealed few commonalities to those from *F. brachygibbosum* (Pradhan, Pandey, Baldwin, personal communication). However, a caveat due to different infected tissues (shoot and root) between the two studies remain. These results support the model of the transcriptomic signal signatures specific for each plant–'attacker' combination [43] and expand the model with the inclusion of miRNAs as part of the signature.

The miRNome signature is context dependent (i.e. microbe-plant combination, environmental conditions, nutrient availability) to allow the plant to mount the appropriate physiological response towards the interacting microbe. miR393 for example, in addition to negatively targeting the auxin signalling, helps the plant to prioritise its resources by re-directing the metabolic flow by stabilising specific ARFs to generate certain anti-microbial compounds (glucosinolates) while repressing others (camalexin) [44]. Thus, miR393 triggers two complementary responses to increase the resistance towards the interacting biotroph *Hyaloperonospora parasitica*, but it renders the plant more susceptible to the necrotroph *Alernaria brasicicola* [44]. Similarly, miR171h, a symbiotic (mycorrhiza and nodulation) induced miRNA [25,27,45] seems to serve as a tool to restrict root colonisation by its symbionts also in a context dependent manner. miR172h targets the GRAS transcription factor NSP2, essential for nodulation and with a role in strigolactone synthesis and in mycorrhizal symbiosis [46–48]. But interestingly, miR172h, which is only found in plants able to form AM symbiosis, is induced by high phosphate under non-symbiotic conditions, showing a negative correlation to its target NSP2 [25,26,45]. During symbiosis, in contrast, both miR172h and NSP2 are induced, albeit with mutually exclusive expression localization patterns [26]. This suggests that in the regulation of miR172h and NSP2 several inputs converge to adjust the mycorrhizal colonisation in a nutrient- and fungal signal-dependent manner [25,26]. Novel roles for miRNAs during symbiotic interactions with AM fungi have been recently discovered, including miRNAs coding for small peptides that regulate their own transcription [17,49], and miRNAs that instead of cleaving their target protect them from cleaving by other miRNAs of the same family [17]. These interesting findings suggest that many yet unknown processes ruling plant–fungal interactions are likely to be fine-tuned by smRNAs.

Achieving the right response with the help of AGO proteins

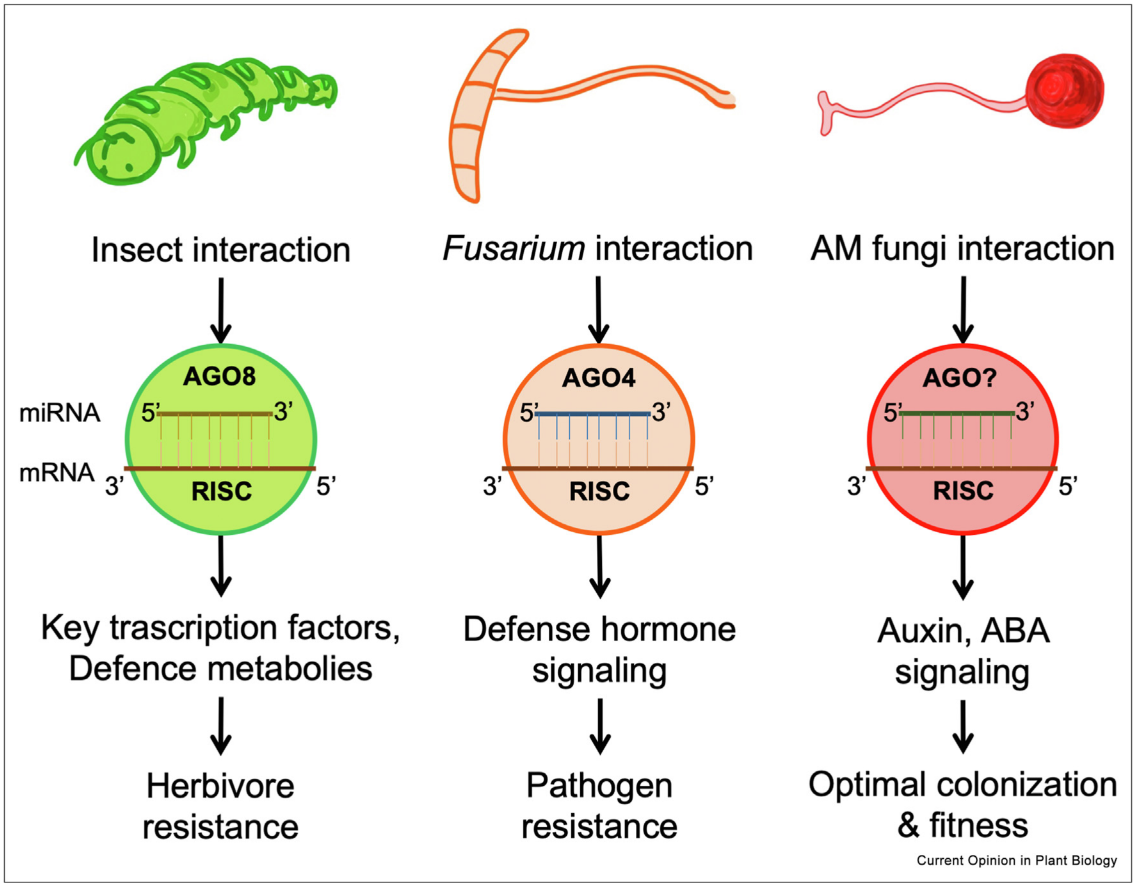
The physiological specificity of miRNome reprograming is achieved via the regulatory function of the RNAi machinery. smRNAs are synthesised with the help of the RdRs and the DCLs [50], and act through the AGOs, making them a core component of this machinery [13]. The AGOs serve as effectors of the pathway, on which miRNAs are loaded to form the RNA-induced silencing complex, RISC [1,51]. Plant genomes code for a large number of AGOs, which could be classified into four evolutionary classes [52]. Analysis of molecular evolutionary patterns suggested that their functional specificity might have arisen due to selection pressure [52], helping to recruit a specific set of miRNAs to evolve novel physiological functions in the context of a plant natural habitat [20,42,53]. Although, how the different AGO proteins are involved in shaping the interaction with specific microbes has not been systematically studied, there are many examples that show that the same AGO protein might play the opposite role on plant susceptibility towards two different microbes and also that the microbe-plant interaction might be preferentially shaped by one or a few specific AGO proteins. Thus, for instance, *Arabidopsis thaliana ago1* mutants exhibit a milder response to bacterial pathogens, but an induced resistance to fungi **[reviewed in 13]**. Also, from the 10 AGO proteins from *A. thaliana*, only AGO2 is induced in response to *Pseudomonas syringae* pv. tomato and consistently *ago2* plants are more susceptible to this bacterium [54]. This suggests that in natural ecosystems the smRNA machinery requires of a coordinated response as plants are simultaneously exposed to different microbes. The ecological functions of the RNAi machinery have been extensively analysed in *N. attenuata* [20,42,55–58]. In that system, it has been shown that the smRNA machinery drives the specificity required for plants to adapt to their natural post-fire habitat by tailoring the appropriate responses to the unpredictable challenges and myriads of competitors ranging from herbivores and pathogens to other individuals from the same species that bloom in that environment [58]. Accordingly, distinct AGO proteins are recruited depending on the nature of the interactor (Figure 1).

smRNAs as major players in plant–fungal interaction at the cross-kingdom level (500 words)

smRNAs have a highly mobile nature, with many of them travelling systemically for short and long distances within plant cells and organs to act in a non-cell-autonomous manner. Some of them are even trafficking between the host and its interacting fungi in a ‘cross-kingdom manner [6]. Interestingly, it has been shown that plant smRNAs can target fungal genes essential for virulence, hence adding smRNAs to the arsenal of defense responses [59,60]. But perhaps even most remarkably, fungi have been shown to hijack the host cell by delivering smRNAs into the plant, compromising immunity [11–15,61]. Such smRNAs act as unique class of fungal effectors that suppress host immune responses. The cross-kingdom movement of smRNAs is thus bidirectional and invokes an interesting conundrum. In addition to fungi sending their smRNAs to hijack host immunity genes, they could also target components of the plant smRNA machinery to promote infection [11,62]. Furthermore, it has been proposed that exogenous smRNAs, if in high abundance, might compete with endogenous smRNAs for loading into the RNA silencing machinery [6]. Thereby, pathogenic smRNAs might in that form indirectly compromise the ability of the infected cells to mount the defense response. This interspecies exchange of smRNAs likely travels in extracellular exosome-like vesicles as demonstrated for plant smRNAs [60,63]. But accumulating evidence suggests that this might be also the vehicle for fungal smRNAs towards the plant [64–66]. Exchange of smRNAs represents consequently an unexpected and extraordinary tool of communication between plants and their interacting organisms.

Although the cross-kingdom action of smRNAs in plant–pathogen interactions has started to unravel rapidly, its role during plant-mutualistic symbiosis is far from being understood. Evidence of smRNAs transition from the plant to the fungus during AM symbiosis exists, as it has been shown that inactivation of fungal genes can be achieved *in planta* by host induced gene silencing, HIGS [67–69]. On the other hand, the genome

Figure 1

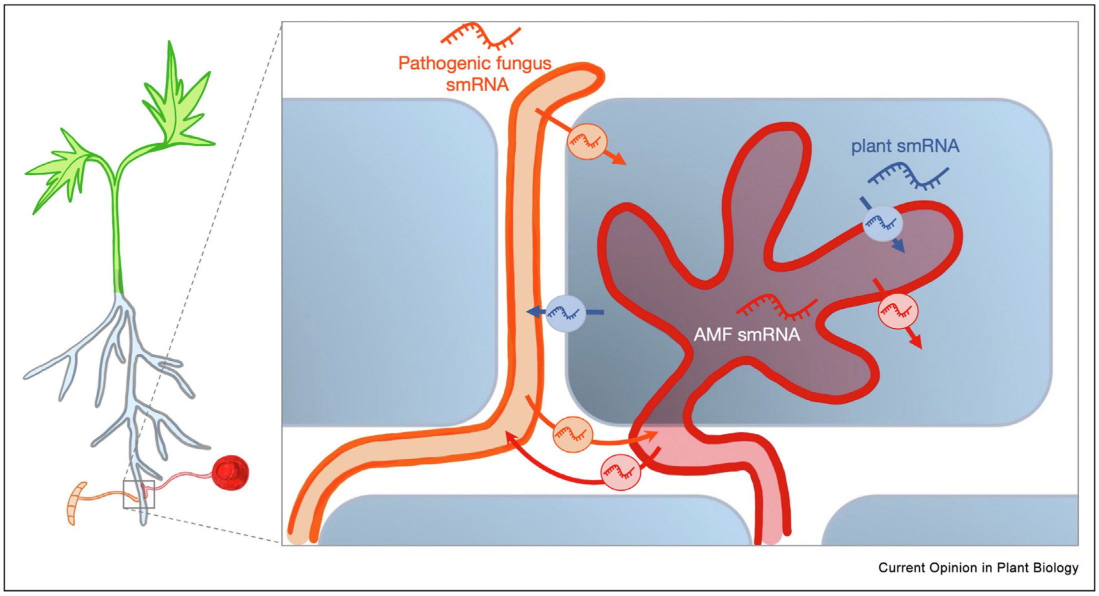


Specialisation of biological function of AGOs during plant interactions with other organisms. AGO proteins are effectors of small RNA pathways as they are loaded by mi/siRNAs, form the RNA-induced silencing complex (RISC) and identify targets in a sequence-specific dependent manner. During plant interactions with other organisms, specific AGO proteins are recruited in the context of the particular interacting organism, that is, an insect (left panel), pathogens (middle panel) or mutualistic AM fungi (right panel). Such specific involvement of AGOs allows distinct portions of miRNome to be recruited to modulate specific responses towards the interacting organism maximising plant fitness. This model has been well studied in *Nicotiana attenuata*. There, herbivores specifically entail an AGO8-mediated smRNA signalling for the production of defense metabolites such as nicotine, phenolamides and diterpenoid glycosides. Accordingly, AGO8 silenced plants are more susceptible to *Manduca sexta* [42]. In contrast, AGO4 is specifically recruited during the interaction of *N. attenuata* with its natural hemi-biotrophic interacting pathogen *Fusarium brachygibbosum* while none of the other AGO protein of *N. attenuata* are required during this interaction [20]. Interestingly, AGO4 is also recruited in tomato in response the wilt pathogen *Fusarium oxysporum* f.sp. *lycopersici* [73]. Similarly, colonisation of AMF is expected to be shaped with the participation of a specific AGO, while none of the other AGOs appears to have a functional role during the symbiotic interaction [58] (Pradhan et al., unpublished data). It is astonishing that plants manage to get a coordinated response to so many different microbial-induced miRNome signatures with a limited number of Argonaute proteins. This suggests that co-evolution of plants and their associated microbes may have posed evolutionary constraints giving rise to the selection of only one or few AGO proteins for one specific microbe.

sequence of several AM fungi has revealed the existence of RdR, DCL and AGO-like genes, suggesting an active smRNA pathway in AM fungi [30,70]. Accordingly, smRNA populations have been identified in *R. irregularis* and their putative targets in the host predicted, some of which with a known role in the AM colonisation [30]. However, they have not been experimentally validated, and thus their biological functions await to be disclosed. Also, in the ectomycorrhizal fungus *Pisolithus microcarpus* a microRNA has been recently identified with a role in symbiosis [29]. Altogether these results suggest a role for smRNAs in shaping fungal mutualistic interactions.

Additional possible scenarios of the involvement of smRNAs in the interaction between plants and their interacting fungi cannot be ruled out. For instance, plants might recognise their interacting fungi by identifying their smRNAs. Furthermore, in natural habitats, pathogenic and mutualistic fungi co-exist and often colonise the same host plant. Thus, it is plausible that smRNAs act in such a complex scenario as communication tools to modulate multi-trophic interactions (Figure 2). It is known that miRNA gradients in plants can help to produce positional information in a group of cells and thus asymmetric distribution of their target

Figure 2



Inter-species and cross-kingdom smRNA exchange during plant–fungal interactions. smRNAs are deployed by plants in response to colonising fungi to adjust not only their own cellular program but also as effectors to communicate and manipulate fungal cells. Fungi also produce smRNA during interactions that travel to plant cells to highjack their defense program. At the same time, we hypothesise that fungal smRNAs might aid plants to discriminate between pathogens and mutualists during multi trophic interactions. A hypothetical transport of smRNAs between different fungal species colonising the same host might help fungi to control their niche. Transport across cell membranes is hypothesised to be conveyed in extracellular vesicles.

(i.e. miR165/156 and its target PHABULOSA in roots), thereby conferring cells a specific identity [71,72]. It would be interesting to investigate whether different fungi co-colonising a plant enable smRNA-governed micro-territories to safeguard their niche. Furthermore, these micro-territories might help plants to quickly integrate different microbial signals and deploy coordinated responses towards natural habitat challenges. These open questions warrant immediate attention.

Concluding remarks

smRNAs are handy tools for plants to discriminate between friends and foes. They regulate signalling during interaction of plants with associating partners and participate in tailoring specificity. The biological function of some conserved miRNAs has diversified across plant lineages possibly as a consequence of the selection pressure that different plant species encounter in their natural habitats [53]. Moreover, as both plants and fungi use smRNAs to communicate and control the cell program of the other partner, this could be critical in defining the nature and outcome of the interaction. But also, smRNAs might function under more complex conditions, helping plants to coordinate responses and endure abiotic and biotic stresses under fluctuating environmental challenges. Additionally, unravelling the identity and function of cross-kingdom-acting smRNAs may provide the much needed tools for biotechnological interventions in agriculture to control pathogenic fungi while promoting mutualistic fungal associations. This will contribute towards a sustainable agriculture by decreasing the need of fungicides and fertilizers.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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Abbreviations

RNAi	RNA Interference
HIGS	Host Induced Gene Silencing
smRNA	small RNA
miRNA	micro RNA
RDR	RNA directed RNA Polymerase
DCL	DICER like Protein
AGO	Argonaute
RISC	RNA Induced Silencing Complex
SA	Salicylic acid
JA	Jasmonic acid

ABA Abscisic acid
AM fungi Arbuscular mycorrhizal fungi

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