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# Small Non-Coding RNAs in Plant Immunity

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Additional information is available at the end of the chapter

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## 1. Introduction

### 1.1. The “zigzag model” of plant-pathogen interactions

During millions of years of co-evolution, plants have established sophisticated genetic mechanisms to protect their integrity against invading pathogens. Pathogens in turn have coped with such barriers to gain access to nutrients and proliferate inside the plant. The “zigzag model” illustrates in a simple way the different layers of innate immunity during interactions of plants with their pathogen [1]. This model describes two main immunity responses, the first one relies on plants' ability to recognize so-called microbial-associated molecular patterns (MAMPs), which are highly conserved structures and molecules in all kinds of pathogenic and nonpathogenic microorganisms. This response is known as MAMP-triggered immunity (MTI) and is efficient against non-adapted or non-host pathogens [2]. The best-studied MAMPs are the flagelline peptide, the elongation factor Tu protein (EF-Tu), chitin which is a major component of fungal cell walls and lipopolysaccharides (LPS). MAMPs perception depends on plant pathogen recognition receptors (PRRs), with FLS2 and EFR recognizing flagelline and EF-Tu, respectively. These two PRRs share a similar structural architecture formed by extracellular Leucine Rich Repeats (LRR) and a cytoplasmic kinase domain. CERK1, on the other hand, which is the Arabidopsis PRR involved in the recognition of chitin, contains three extracellular LysM domains and a cytoplasmic kinase domain. In response to MTI, pathogens developed strategies to overcome it by sending effector proteins inside plant cells. These effector proteins abolish MTI by either suppressing early recognition or interfering with down-stream signaling events [3]. A second layer of plant immunity known as effector-triggered immunity (ETI) relies on a more sophisticated mechanism to detect pathogens, based on the specific recognition of particular effector proteins by Resistance (R) proteins. In this case the effector proteins are named Avr factors. The Avr-R proteins interaction can be direct or mediated by another protein, referred to as pathogenicity target. In the later case the R protein guards the pathogenicity target and detects its modification caused by the effector protein [4]. The

largest group of R proteins includes a Nucleotide Binding Site (NBS) and a LRR domain. This group can be subdivided in two subclasses based on the presence or not of a Toll-Interleukin Related (TIR) domain in the N terminus. Upon the perception of pathogen's molecules by PRRs or R proteins, a signaling cascade including MAP kinases is activated, leading to a reprogramming in host's gene expression along with the activation of genes with antimicrobial function (PR, pathogenesis related) [2, 5]. In the last years a great effort in research has focused on understanding how gene expression is modified in response to pathogens, revealing some crucial factors for the interaction such as transcription factors, DNA regulatory elements and non-coding small RNAs.

## 2. An overview of beneficial interactions

Plant-microbe interactions are not always disadvantageous to plants. During millions of years of co-evolution, plants established symbiotic interactions with bacteria and fungi. The best-studied models illustrating this kind of interaction are the rhizobial and mycorrhizal symbiosis, which involve a particular group of bacteria and fungi, respectively. The establishment of symbiosis requires a concerted molecular dialogue involving the correct recognition and the activation/repression of specific signaling pathways [6]. In the rhizobial symbiosis, *Rhizobia* form an intimate relationship with leguminous plants. Plants provide carbon and energy to the bacteria, that in exchange fix atmospheric nitrogen of interest for plants [7]. Compatible *Rhizobium* species perceive plant-secreted flavonoids and induce the expression of bacterial *nod* genes that are essential for the development of nodules in plant hosts. The Nod factors are recognized by specific plant receptors carrying an extracellular LysM domain and an intracellular kinase domain. Upon perception several cytoplasmic events occur at the root epidermal cells, including membrane depolarization, calcium spiking and activation of a calmodulin-dependent kinase signaling [7]. These processes create a favorable cellular environment leading to the establishment of an infection thread branch through which *Rhizobia* penetrate into the host. Once in the cytoplasm, bacteria group and form bacteroids where nitrogen fixation occurs. In contrast to this highly specific interaction between legumes and *Rhizobia*, mycorrhizal fungi establish symbiosis with almost all terrestrial plant species. In this case, fungi provide nutrients from the soil to the plant, particularly P, and in exchange plants feed the fungus with their photosynthetic products [8]. The nutrient transfer occurs in the arbuscules, which are specialized structures formed in cortical root cells. Arbuscular mycorrhizae (AM) depend on the activation of a symbiosis signaling (Sym) pathway, which shares some elements with rhizobial symbiosis [6]. In the case of fungal AM, the perception relies on the recognition of diffusible Myc factors, leading to the reprogramming of the basic metabolism of plant cells and hyphens.

However, these types of beneficial interactions are not always successful. Plants indeed tolerate the invasion of these microorganisms only under nitrogen or nutrient-deficient conditions. In consequence, a sophisticated perception mechanism should exist in plants in order to simultaneously estimate nutrient deficiency and distinguish between beneficial microbes and pathogens. To achieve symbiosis, a fine regulation of the plant immune responses is therefore required for accepting or not candidate microorganisms [6]. In the last

years we have learned a lot about how non coding RNAs are a crucial players in regulating such responses as well others.

### 3. Non-coding small RNAs

In eukaryotes, the endogenous regulation of gene expression is mostly dependent on the control of the RNA polymerase II by accessory proteins including activators, repressors and the mediator complex. Then, small non-coding RNAs (sncRNA) were discovered and found to be new key elements of gene expression regulation [9, 10]. sncRNAs are short molecules of typically 18 to 30 nt, involved in gene expression control, defense against other parasitic nucleic acids, epigenetic modification and heterochromatin regulation.

The best-studied sncRNAs are microRNAs (miRNAs) and small interference RNAs (siRNAs). miRNAs derive from nuclear genes. A gene coding for a miRNA (*MIRNA*) is first transcribed by the RNA polymerase II to a primary miRNA (pri-miRNA), the size of which ranges from 100 nt to several kilobases (kb). A Dicer-like (DCL) protein DCL1 in *Arabidopsis* along with *HYPONASTIC LEAVES1* (*HYL1*), process the pri-miRNA into a 70 to 400 nt long precursor miRNA (pre-miRNA). This pre-miRNA forms a characteristic hairpin-like structure. A subsequent processing step involving DCL slices the pre-miRNA to form a miRNA:miRNA\* duplex (21-22 nt). The duplex is then methylated by HEN1 and exported from the nucleus to the cytoplasm where it will join an AGO protein to form the silencing complex (RISC). Only the mature miRNA strand which is usually the one with less stable 5'-end pairing, is retained in the complex, while the passenger (miRNA\*) strand is degraded. The miRNA\* degradation process remain unknown, although some family of exoribonucleases encoded by the *SMALL RNA DEGRADING NUCLEASE* (*SDN*) genes degrades mature miRNAs which could also be involved in the miRNA\* degradation [11]. The miRNA retained in the RISC complex will then guide the silencing of complementary mRNAs (targets) [12-13].

In contrast, siRNAs originate from transgenes, viruses, transposons or other RNAs that form perfectly complementary double-stranded RNA precursors (dsRNAs). In particular, virus-derived siRNAs also known as virus induced RNAs (vsiRNAs), have been extensively studied in plants. From a siRNA precursor, multiple siRNAs are generated and the silencing signal can be further amplified upon the generation of secondary siRNAs subsequently processed by RNA dependent RNA polymerases (RDRs), *SILENCING DEFECTIVE3* (*SDE3*), *NRPD1a* and *NRPD1b* (largest subunits of Pol IVa and Pol IVb isoforms of RNA polymerase IV, respectively) [14-16].

Recently, various new types of ncRNAs have been described. Among them are the trans-acting siRNAs (ta-siRNAs) (21-22nt) which combine both the siRNA and miRNA pathways since they originate from a nuclear *TAS* gene which is transcribed into a mRNA and cleaved by a miRNA. The cleaved product is converted into dsRNA by the *DEFECTIVE IN RNA-DIRECTED DNA METHYLATION/SUPPRESSOR OF GENE SILENCING* (*RDR6/SGS3*) processing complex, and leading to specific siRNAs called ta-siRNAs. These mature 21-nt long siRNAs, similar to miRNAs, are able to initiate the cleavage of homologous cellular

transcripts, thus acting in trans. Additionally the siRNA signal from ta-siRNAs can also be amplified upon the generation of secondary siRNAs [14, 17-19]. Also reported are natural antisense transcripts-derived siRNAs (nat-siRNAs) (21-24 nt) which are cis-acting siRNAs derived from naturally occurring overlapping regions of sense and antisense transcripts [20]. The long siRNAs (lsiRNAs) (30-40 nt) are DCL1 and AGO7 dependent in their biogenesis and act by decapping or by 5'-3' degradation of target mRNAs [21]. Whilst other types of sncRNA exist, we will focus on the most common ones and report on their role(s) in plant immunity. A comparison of the types of sncRNAs discussed in this chapter can be seen in table 1.

	siRNA	miRNA	ta-siRNA	nat-siRNAs
Derived from	Invasive nucleic acids (virus, transgenes)	non-coding regions	non-coding regions	antisense genes
Transcribed by	Depends of origin	RNA pol II	RNA pol II	RNA pol II
Processed by	DCL, RDR, SDE, NRPD	DCL1, HYL1, HEN1	RDR6/SGS3, DCL, miRNAs	DCL1, HYL1
Targets transcripts in	cis	trans	trans	cis
Binds to	AGO1, AGO2	AGO1	AGO1, AGO7	AGO1?

**Table 1.** Comparison of important features of common types of sncRNAs found in plants.

#### 4. The “zigzag model” in plant-virus interactions

The seminal work achieved on plant-virus interactions studies led to the discovery of post-transcriptional gene silencing (PTGS) as a genuine plant defense mechanism against virus. Most of the plant viruses are positive single stranded RNAs (ssRNAs). To colonize and multiply into new plant cells, virus have to replicate several thousands of times. During this process of replication and infection, RNA viruses produce double-stranded RNAs (dsRNAs). A DCL protein, usually DCL4 in Arabidopsis, recognizes these dsRNAs and cleaves them producing vsiRNAs. The vsiRNAs are next incorporated into the RNA-Induced Silencing Complex (RISC) where only one of the two RNA strands is retained. This RNA strand is complementary to the viral RNA and exploited to target the RNA viral molecule and degrade it. Some vsiRNAs serve as template and substrate of an RNA-dependent RNA polymerase (RdRP), thereby amplifying the signal and producing more vsiRNAs [16, 22, 23]. These vsiRNAs move through the plasmodesmata of the cell-cell assuring a systemic anti-viral defense response [24, 25]. As a general plant defense mechanism against all viruses, this first branch of resistance can be considered as analogous to MAMP-triggered immunity. In this case, dsRNAs are considered as MAMPs and DCLs that recognize the dsRNAs are viewed as a sort of PRR.

In line with the “zigzag model”, virus evolved strategies to overcome this first layer of immunity. As a matter of fact, viruses carry silencing suppressors (SS) are able to act at different levels of the silencing pathway [26]. Considering these suppressors as bona fide



pathogen effector proteins, this scenario is reminiscent of effector-triggered susceptibility or ETS. SS proteins were previously considered as pathogenicity factors with an important function in the development of symptoms on plant hosts during infection [26]. A single SS can exert suppressor activity at different steps of the silencing pathways. One of the best-studied and more versatile SS is HC-Pro which is able to block silencing by either interfering with DCL proteins [27] and/or sequestering 21-nt siRNA duplexes [28]. The TCV P38 coat protein [29] and CMV 2b protein [30] affect the processing of dsRNA through the inactivation of DCL proteins. The P21 and P19 proteins respectively produced by the Beet yellows virus (BYV; Closterovirus, Closteroviridae) and the Tomato bushy stunt virus (TBSV, Tombusvirus, Tombusviridae), exert their function by interacting with miRNA duplexes and hairpin derived siRNAs [28, 31, 32]. Additionally, these SS interfere with the small RNAs stability by blocking the activity of the methyltransferase protein HEN1 [33, 34]. The Beet western yellows virus (BWYV; Polerovirus, Luteoviridae) P0 protein interacts via an F-box domain with AGO1 which results in its degradation, illustrating again how important components of the RNA silencing machinery are targeted in order to affect silencing [35].

To overcome virus-deployed strategies suppressing silencing, plants evolved R proteins recognizing specific viral proteins to trigger an immune response, which can be considered as a sort of effector-triggered immunity. This R-protein dependent ETI depends on the recognition of so called Avr proteins, which can virtually be encoded by any viral coding-gene. Examples of viral Avr proteins include the coat, helicase, replicase and movement proteins [36]. More than 15 anti-viral R proteins and belonging to the large class of NBS-LRRs have been characterized, including R proteins N [37], Y-1 [38] and RT4-4 [39] respectively isolated from *Nicotiana tabacum*, *Solanum tuberosum* and *Phaseolus vulgaris* and conferring resistance to TMV, PVY and CMV. On the other hand, the Rx [40], HRT [41] and RCY1 [42] proteins respectively isolated from *Solanum andigena* and Arabidopsis, belong to the CC-NBS-LRR sub-class. The immunity triggered by these proteins is considered as monogenic and dominant, and manifested by an hypersensitive response (HR) [43]. Interestingly in most cases resistance of plants against virus segregates as a recessive trait, and is expressed as a cellular immunity. Remarkably, all recessive resistance genes isolated so far encode translation initiation factors [36]. As mentioned before, once the recognition of the virus is established, a re-programming in host gene expression takes place in plant host cells [44-47]

## 5. sncRNAs and viruses: new frontiers of defense

Recent studies suggest that sncRNAs are involved in global gene expression changes during plant-virus interaction. It has been proposed that the expression of plant miRNAs targeting plant transcripts is altered in response to virus recognition with the aim of affecting viral replication and spreading. Indeed various plant miRNAs are known to be up- or down-regulated following viral infection [48-52]. For example miR1885 is induced in response to infection of *Brassica rapa* by Turnip mosaic virus and is known to target a TIR-NBS-LRR (TNL) disease-resistance gene [53]. miR164 is also induced upon viral infection and its

induction is due to hormone-dependent specific transcriptional activation [54]. However, the effect of this differential regulation in the outcome of the interaction is not well established, as it may also result from the silencing suppression activity of the virus. This is also the case of miR168, which is induced upon infection by various viruses in different plants [49, 55]. It was shown recently that miR168 accumulation upon infection with Cymbidium ringspot virus (CymRSV) is due to the action of the p19 SS [56]. The specific targeting of miR168 by p19 may be crucial for viral infection given that this miRNA is involved in a regulatory loop with AGO1, which forms the RISC complex and in thus involved in various silencing processes [56].

It has also been suggested that miRNAs directly target viral RNAs, as it occurs in animals [57, 58]. Indeed several studies demonstrated that artificial miRNAs (amiRNAs) targeting key components of the viral replication machinery can efficiently impair viral growth upon infection [59-65]. These efforts have revealed that such resistance is cell-autonomous, inheritable, more efficient than siRNA-mediated strategies and successful in blocking viral replication and movement [59, 66]. Furthermore transgenic plants expressing dimeric or polycistronic amiRNAs directed against different viruses result in a wider spectrum of viral resistance [60, 61, 63].

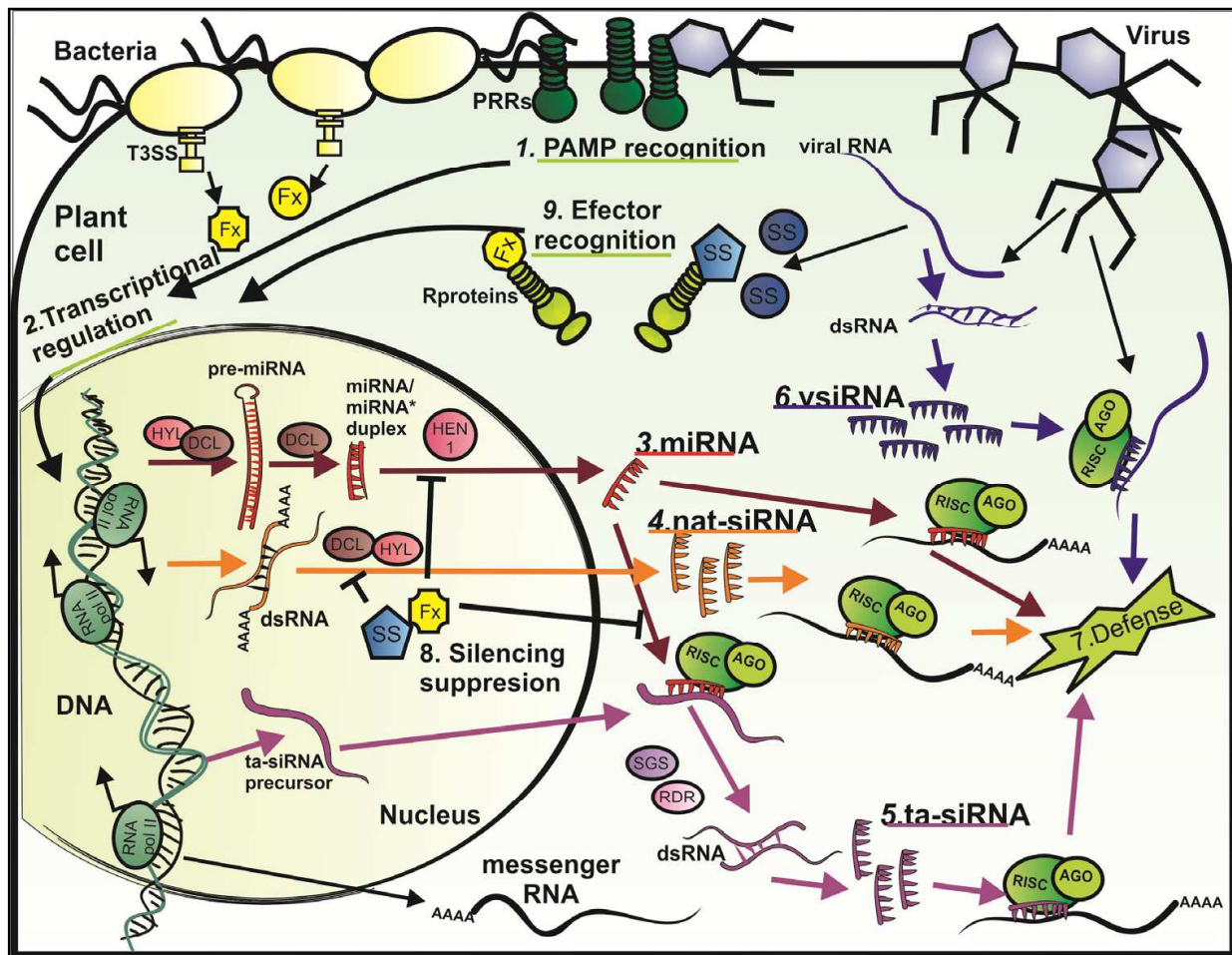
The hypothesis of plant microRNAs naturally evolving to target viral genomes has long been discussed, taking in account the potential disadvantages of the miRNA pathway over the siRNA one. The miRNA pathway is not an adaptive response since the evolution of viral genomes would be fast enough to surpass the evolution of miRNAs rendering them ineffective in a very short term. However some miRNA families may be adapted to target viral genomes, as suggested by bioinformatic analysis [67, 68].

Viruses also encode miRNAs (or similar ncRNAs) directed against the plant genomes (or even their own genomes), that will use the host miRNA machinery to be processed and execute their silencing effect. This mechanism has been described only in animal infecting viruses [69]. So far, the only mechanisms found resembling viral miRNAs in plant viruses refer to sRNAs encoded by the Cauliflower mosaic virus (CaMV) that are partially complementary to regions of the Arabidopsis genome [70] and viral sRNAs that bind the RNAi machinery to divert the silencing machinery from viral promoter and coding regions [71].

A general model of the way sncRNAs mediate the interaction between plants and viruses (as well as with bacteria discussed below) can be seen in Figure 1.

## **6. The need for auxin: responses to bacteria, fungi and symbiotic microbes**

Auxin is a relatively well-known plant hormone mainly implicated in growth which acts, under particular conditions, as a repressor of salicylic acid (SA). SA is a hormone involved in the activation of plant defenses in response to biotrophic pathogens [72, 73]. It is therefore not surprising that plants, in response to microbes, have evolved sophisticated mechanism for fine-tuning of SA-mediated responses.



**Figure 1.** General model of sncRNAs role in plant-pathogen interaction. 1. Upon recognition of PAMPs from virus and bacteria the 2. transcription of loci coding for sncRNAs is regulated so the biogenesis pathways of either 4. miRNAs 5. nat-siRNAs 6. ta-siRNAs is activated with the aims of 7. targeting genes that when silenced would trigger defense responses (like Auxin response factors). Transcription of sncRNAs could be stopped by the plant in response to pathogens so that positive regulators of immunity (like NBS-LRR proteins) can escape miRNA regulation (not shown). 8. Additionally, in the case of virus, vsiRNAs can be produced during viral replication and they would target viral RNAs thus producing defense. 9. Virus and bacteria can counterattack by using effectors and silencing suppressors to disrupt silencing and in response 10. plants could recognize these effectors via resistance proteins. Fx= bacterial effector, SS= silencing supresor, dsRNA= double-stranded RNA, PRRs= pathogen recognition receptors, R proteins = resistance protein, RISC= RNA-induced silencing complex

The best-studied miRNA induced upon bacterial infection is miR393. By comparing the gene expression profile of wild type and transgenic plants expressing several viral SS, it was elegantly demonstrated that upon treatment with flagellin (flg22), some transcripts were more abundant in transgenic plants. Among them was found a transcript coding for the F-box auxin receptor TIR1. By RACE (rapid amplification of cDNA ends) the authors demonstrated that this particular mRNA is targeted and cleaved by miR393. The perception of flagellin by plants induced the expression of miR393 which correlates with a clear reduction of the TIR1 protein content. This led in turn to the stabilization of Aux/IAA proteins which repress auxin signaling by heterodimerization with Auxin Response Factors



(ARF). Flagellin perception leads to a repression of auxin signaling and consequently restricted the growth of *Pseudomonas syringae* pv. tomato (*Pst*). This study provided for the first time a link between auxin response, miRNAs and MTI. In summary plants repress the auxin signaling pathway in response to bacterial hit, favoring the defenses activated by SA, compromising vegetative growth [74].

miR393 was shown to be induced in Arabidopsis plants inoculated with *Pst* DC3000 strain mutated in *hrcC* [75]. This strain is defective for type III secretion, unable to deliver effector proteins into the host plant cell and consequently triggers MTI. Employing a small-RNA profiling analysis, Zhang *et al.* [76] investigated the differential expression of miRNAs in plants challenged with *Pst* DC3000 *hrcC* mutant, a virulent strain of the same species carrying an empty vector and avirulent *Pst* DC3000 containing the *avrRpt2* effector [76]. Curiously, miR393 was repressed at 6 hours post-infection (hpi) and induced at 14 hpi in the three treatments. However Northern-blot experiments show an induction in all treatments and at both time points. To explain this discrepancy, the authors suggest that miR393 may regulate auxin signaling at an early stage of bacterial infection [76].

The complex interplay between auxin and miRNAs goes beyond miR393. Several reports have shown that different ARFs and auxin receptors coding genes are regulated by other miRNAs such as miR160, miR166 and miR167, not only in response to phytopathogenic bacteria and fungi but also during beneficial interactions involving *Rhizobium* or AM [72, 76-80]. MiR167 and miR160 which target ARFs genes, are induced upon infection with different *Pst* DC3000 strains [76, 77]. MiR160 was also found to be induced in response to flg22 and bound to AGO1. Transgenic plants over-expressing miR160 show enhanced callose deposition and higher resistance to DC3000 indicating a role for miR160 as positive regulator of plant pathogen response [78].

miR393 is highly conserved and was also detected in cassava plants challenged with *Xanthomonas axonopodis* pv. manihotis, which is the causal agent of Cassava Bacterial Blight (CBB) [81]. Interestingly the expression of miR160 and miR393 is reduced during the infection of Arabidopsis with *Agrobacterium tumefaciens*, thereby increasing auxin signaling [82].

During the symbiosis occurring between soybean and *Bradyrhizobium japonicum*, miR160, which targets the auxin repressor ARF17, is down-regulated, suggesting an increase in free auxin during this interaction. In contrast, miR393 was found to be induced, which is in opposition to miR160 effect, as miR393 regulates the auxin receptor TIR1 and consequently inhibits auxin signaling [83]. In AM symbiosis, a strong connection between miRNAs and auxin has also been unveiled. During the interaction of *M. truncatula* plants with *Glomus intraradices*, it was reported that miR160c and miR167 are induced in mycorrhizal roots while miR160 was predominantly localized in the phloem [84]. These authors reported also miR5229a/b to be the most induced miRNA. By in situ hybridization it was demonstrated that it was exclusively expressed in arbuscule-containing cells of the root cortex, albeit with different signal intensities indicating a specific function during different stages of the arbuscule development. The predicted target of miR5229a/b is a transcript encoding for a heme peroxidase playing different roles in the regulation of ROS production, cell wall biosynthesis but also auxin and ethylene metabolism. This provides another example of the

relationship between miRNAs and auxin at a more indirect level [84]. Among 33 pathogen-responsive miRNAs detected during the interaction of *Populus beijingensis* with the fungus *Dothiorella gregaria*, the induction of miR393 was observed with a peak of expression at 7 days after inoculation but their levels were reduced at 14 and 21 days [85]. On the other hand, the same study showed the repression of miR160 as was previously reported in pine infected with the fungus *Cronartium quercuum* f. sp. *fusiforme* [86]. In wheat interaction with *Erysiphe graminis* f. sp. *tritici*, the expression pattern of miR393 was less expressed in the susceptible cultivar Jingdong8 as compared to the near-isogenic resistant line Jingdong8-Pm30 [87]. These results illustrate the fact that in some cases, conserved miRNAs may play similar functions in different pathosystems. However in other cases, the expression profile and in consequence the function of miRNAs can be specific even if their sequences and targets are conserved. Altogether, these results highlight how crucial is auxin balance in plant-microbe interactions.

## 7. MTI and silencing: beyond miR393 and auxin

Fahlgreen *et al.* [77] reported on miRNAs repressed upon infection with a strain of *Pst* DC3000 inactivated in *hrcC* which is a major component of the TTSS. One of the identified miRNAs is miR825, which is predicted to target transcripts encoding a Remorin, a transcription factor of the zinc-finger homeobox family and a frataxin-related protein. These targets are known to act as positive regulators of plant defense, it is therefore expected that the miRNAs controlling them are repressed.

A first connection between miRNAs-mediated silencing and MTI emerged from the study of Arabidopsis AGO1 mutant lines, found to be compromised in MTI [78]. These plants are characterized by a reduction in seedling growth inhibition, callose deposition, expression of MTI-markers genes and the activation of MAP kinases and ROS production upon treatment with flagellin. Also, the growth of TTSS-mutant strains was increased in these plants. These results demonstrate that AGO1, and indirectly the silencing pathway, are key elements of MTI. These phenotypes are not observed in AGO7 mutant plants, indicating that only AGO1 activity is associated with MTI. Interestingly, the involvement of miRNAs in regulating plant immunity is not restricted to MTI. It may also be associated to ETI, as exemplified by cases of plant resistance responses against fungal pathogens, where several components of the silencing machinery were showed to play a role [88]. More precisely, Arabidopsis *sgs2-1*, *sgs1-1* and *sgs-3* mutants which are defective in siRNA production, are more susceptible to various strains of *Verticillium dahliae* but not to other pathogens such as *Botrytis cinerea*, *Alternaria brassicicola* and *Plectosphaerella cucumerina*. Intriguingly, while *ago7-2*, *dcl4-2*, *nrdp1a-3* and *rdr2-4* mutants are more susceptible to *V. dahliae*, *ago1-25*, *ago1-27*, *hen1-6* and *hst-1* mutants display enhanced resistance to this pathogen. Finally, *dcl-2*, *sde3-4* and *sde3-5* mutants were as susceptible as the Arabidopsis Col-0 wild type line [88].

## 8. Towards ETS

Accordingly to the “zigzag model”, adapted pathogens overcome MTI to infect particular host plants. Based on the study of Arabidopsis mutants affected in genes involved in the

silencing pathway like *dcl* and *hen1*, it could be demonstrated that non-pathogenic bacteria like *P. fluorescens* and *E. coli* were able to grow in these plants and not in the wild type. In addition, an increase in growth was observed upon inoculation of silencing-defective mutant lines with a TTSS-mutant *Pst* DC3000 strain and the non-host pathogen *Pseudomonas phaseolicola* [89]. These ground-breaking observations suggested a pivotal role of silencing in triggering MTI responses. In consequence, adapted pathogens should have acquired effector proteins capable of suppressing silencing-associated MTI pathways. As a matter of fact, the virulent strain *Pst* DC3000 was reported to repress the expression of miR393 which is normally induced upon flagellin recognition as well as other MTI responses, whereas a TTSS-mutant strain did not [89]. Since virulent *Pst* DC3000 wild type strain harbors an intact TTSS, it was concluded that miR393 repression results of the action of injected effectors. Upon *Agrobacterium*-mediated transient expression of particular T3 effectors into *Arabidopsis* leaves, the expression of miR393 primary transcripts was monitored. This assay successfully demonstrated that T3Es AvrPto and HOPT-1 block the miRNA pathway by targeting the activity of DCL1 and AGO, respectively [89]. Interestingly, it was also demonstrated that pre-inoculation of virus containing SS led to the development of disease-like symptoms and favored multiplication of non-pathogenic and non-host bacteria inoculated subsequently. The authors suggest this as a molecular base explaining the synergistic interactions eventually observed between some viral and bacterial phytopathogens in the field [89].

## 9. The arms race goes on: miRNA's role in ETI

As mentioned previously, the specific recognition of effectors by R proteins triggers ETI, which involves gene expression reprogramming. In a survey aimed at determining the role of siRNAs in gene expression during R-protein-mediated responses it was found that a 22 nt nat-siRNA was induced specifically by *Pst* DC3000 containing the avirulence gene *avrRpt2* [90]. This nat-siRNA named nat-siRNAATGB2 is produced due to an overlapping region between the *At4g35860* and *At4g35850* transcripts. *At4g35860* encodes a Rab2-like small GTP-binding (*ATGB2*) while *At4g35850* encodes a PPR (pentatricopeptide repeats) protein-like gene (*PPRL*). The sequence of the nat-siRNAATGB2 is complementary to the 3' UTR region of the antisense gene *PPRL*. In fact it was demonstrated a correlation between the induced expression of nat-siRNAATGB2 and a repression of *PPRL* after infection with *Pst* carrying *avrRpt2* [90]. The induction of nat-siRNAATGB2 is dependent of the presence of *RPS2* and *NDR1*, two genes required for the induction of the *avrRpt2*-mediated response. The biogenesis of nat-siRNAATGB2 depends on the DCL1-HYL1 complex, which is stabilized upon HEN1-mediated methylation and amplified by RDR6 and SGS3. In concordance, plants mutated in these genes do not show a reduction of the *PPRL* expression. On the other hand, the overexpression of *PPRL* produced a delayed HR and enhanced growth of *Pst* DC3000 carrying *avrRpt2*, indicating that *PPRL* is a negative regulator of plant defense responses [90]. A novel class of sncRNAs induced in response to *Pst* DC3000 strain carrying *avrRpt2* was also identified [21]. In this case the sncRNAs are long siRNA (lsiRNA) of 30 to 40 nt. Among these lsiRNAs is AtlsiRNA-1 which is generated from a NAT pair

between the genes *SRRLK* and *AtRAP*. Interestingly, the biogenesis of *AtlsiRNA-1* requires *DCL1*, *DCL4*, *AGO7*, *HYL1*, *HEN1*, *HST1*, *RDR6* and *Pol IV*. The target of *AtlsiRNA-1* is the gene *AtRAP*, which encodes for a RAP-domain protein with a role in plant resistance to pathogens. *AtlsiRNA-1* does not cleave its target mRNA as most siRNAs usually do, but it guides their degradation through decapping and *XRN4*-mediated 5'-to-3' decay. A knockout mutation in *AtRAP* increases the resistance of *Arabidopsis* against virulent and avirulent *Pst* strains. In addition, overexpression of *AtRAP* leads to an increase in bacterial growth [91]. Thus, *AtRAP* and *PPRL* can be considered negative regulators of plant immunity.

## 10. R genes, my favorite targets: miRNAs

Since the expression of *R* genes is constitutive in most cases, it should be expected that plants have developed mechanisms regulating their activity and restrain the activation of plant immune responses under pathogen-free conditions. Although an elegant mechanism of regulation of NB-LRR proteins by conformational changes depending of the presence of the effector and hydrolysis of ATP has been described [92], controlling the activity of negative regulators mediated by sncRNAs emerges as an additional strategy to control plant immune responses.

Some reports have indeed demonstrated a direct regulation of *R* genes by siRNAs. In a pioneering study, Yi and Richards [93] detected endogenous siRNAs at the *RPP5* locus with antisense transcription activity. In this locus were identified seven *R* genes of the TNL class interspersed with three related sequences and two other non *R* genes. The genes *RPP4* (Recognition of *Peronospora parasitica*, now referred to as *Hyaloperonospora arabidopsis*) and *SNC1* (suppressor of *npr1-1*, constitutive 1) present in this cluster confer resistance to fungal and bacterial pathogens, respectively and are coordinately regulated by transcription control. It was shown that a production of antisense transcripts generates siRNAs to regulate the mRNA level of these genes. In fact, in *dcl4* and *ago1* mutant *Arabidopsis* plants the expression of *SNC1* mRNA was elevated suggesting a role of siRNAs involved in its regulation [93]. A similar observation was reported in the symbiotic interaction of *M. truncatula* with *Shinorhizobium meliloti* where genome-wide analysis of small RNAs revealed a relatively high proportion of 21-nt sRNAs corresponding to NBS-LRR genes [94].

Another example of regulation of *R* genes mediated by sncRNAs deals with the tobacco *N* gene which confers resistance to the tobacco mosaic virus (TMV) and codes for a TNL protein. Two miRNAs were shown to guide the cleavage of the *N* gene, namely *nta-miR6019* and *nta-miR6020* of 22 and 21 nt-long, respectively. In addition, a production of secondary siRNAs "in phase" with the *miR6019* cleavage site of the *N* gene transcript was evidenced, and their biogenesis is dependent on *DCL4* and *RDR6* [95]. The co-expression of *N* with both of these *nat-miRNAs* led to reduced resistance against TMV confirming the importance of these *nat-miRNA* in the regulation of the *N* gene and *N*-dependent immune responses. The authors expanded these discoveries to tomato and potato, two species of the same Solanaceae family, finding that members of these miRNAs families are conserved across species as well as their potential for cleavage of NBS-LRR transcription products and the generation of secondary siRNAs [95].



During the infection of pine with the fusiform rust fungus *Cronartium quercuum*, a ta-siRNA (pta-22 ta-siRNA) targeting two disease resistance proteins was identified. In addition this study also reported and validated experimentally pta-miR946 and pta-miR948 and six of their targets which are predicted to encode for disease resistance-related transcripts, a transcript with similarity to *RPS2* and serine/treonine kinases [86]. Two other miRNAs (pta-miR950 and miR951) also target *R* genes [86]. In the response of poplar to the fungus *Dothiorella gregaria*, the targets of miRNAs pbe-miR482b, pbeSR3, pbe-SR23 and pbe-SR25 also include *R* genes. Other miRNAs previously identified in *Populus trichocarpa* include miR1447 which targets a related disease resistance-coding gene, while two other conserved miRNAs also targeting *R* genes (miR1447 and miRNA1448) are repressed [85]. Once again, these results highlight a complex network of several *R* genes whose regulation is coordinated by a huge collection of miRNAs belonging to different families and isoforms. Although miRNAs and their targets are not always validated experimentally, there is overall a clear consistency between the expression of disease resistance-related genes and their corresponding miRNAs.

*R* proteins play a pivotal role in triggering immune responses and should be able to recognize a broad spectrum of effector proteins (Jones and Dangl, 2006). The high repertory of plant immunity genes raises the question as to the control of their activity. Not surprisingly, several mechanisms were reported explaining the regulation of the expression and activity of this important type of genes. Because of the constitutive nature of many resistance genes expression, fitness costs translating into reduction of growth and productivity are significant. The regulation of *R* genes by miRNAs could have evolved as an alternative strategy for tight and cost effective regulation.

On the other hand, to achieve successful colonization, symbiotic microbes must be able to block plant immune responses triggered by non-self recognition. An expected strategy would be through the control of plant immunity master regulators, such as *R* genes-encoded proteins and other immunity receptors. As a matter of fact, miR482 was reported to be induced during the establishment of symbiosis between soybean and *Bradyrhizobium japonicum*. Interestingly, bioinformatically-predicted targets of miR482 include various *R* genes of which two were validated experimentally. In addition, a considerable increase in the number of mature nodules was observed upon accumulation of miR482 conditionally expressed in roots under a *Rhizobium*-responsive promoter [96]. In *M. truncatula* challenged with *Shinorhizobium meliloti*, 14 targets predicted for 9 Mtr-miRNA candidates correspond to NBS-LRR coding genes [94]. Also, a high proportion of targets identified in a degradome library generated from *M. truncatula* plants infected with *Glomus intraradices* include *R* genes (27 genes) and transcription factors (33 genes). In particular it was established that miR1510a\*, miR1507, miR2678 and miR5213 regulate the expression of a subset of *R* genes [84]. More recently a deep sequencing analysis of 21 sRNAs libraries generated from four legumes (*M. truncatula*, soybean, peanut and common bean) led to the identification of several phased siRNAs (potentially ta-siRNAs), most of them targeting NBS-LRR encoding genes. These findings were expanded to potato based on bioinformatic analysis [97]. Although none of the phased siRNAs were validated by alternative experiments, the deep sequencing and the high number of libraries support well these data.



Besides disease resistance-related genes, other genes involved in defense pathways signaling are regulated by miRNAs. In the above-mentioned study of *Pinus taeda* infected with the fusiform rust, potential targets of a few isolated miRNAs include transcripts encoding a MYB transcription factor (pta-miR159), laccase-like genes, (miR397), peroxidases (miR420) and glutation S-transferase (GST). All these genes play a role in plant responses to pathogens, notably in gene regulation and control of ROS production [86]. Consistently, it has been shown that some of these targets are also regulated by miRNAs to avoid their expression during symbiosis. miR5282 and miRc\_275 were induced specifically in mycorrhizal roots. These miRNAs both target *MtGst1* which encodes for a GST [84].

In conclusion it appears obvious that a successful symbiosis requires suppression of host defenses. Altogether these reports stress that *R* genes are set under a multilayered and complex regulation network during interactions with microorganisms, meant to allow the establishment of beneficial interactions in favorable conditions and avoid in the mean time the invasion of pathogens.

## 11. Novel and specific miRNAs in beneficial interactions

sRNA studies for beneficial interactions have focused mainly on the study of legumes, and had benefited from the identification of sRNA loci in model legumes as *Lotus*, *Medicago*, *Glycine* and *Phaseolus* [98-100]. Some studies focused on the expression of specifically-induced or repressed miRNAs during symbiosis at early [83] or late stages of the infection [72, 94]. For example, during the infection of soybean with *Bradyrhizobium japonicum*, miR168 and miR172 were induced during the first 3 hours but were gradually down regulated to reach basal levels at 12 hours. In contrast, the induction of miR159 and miR393 was sustained along the 12 hours, whereas miR160 and miR169 were down-regulated [83]. Interestingly, these studies allowed the identification of apparently specific miRNAs present or expressed only in plants able to form AM or in the symbiotic structures, respectively. Among the soybean miRNAs identified during interaction with *B. japonicum*, miR1507 seems to be legume-specific whereas miR1512, miR1515 and miR1521 were only reported in soybean [96].

miRNA expression was studied in soybean mutants *nod49* (mutant for a Nod factor receptor *NFR1*) and *nts382* (mutant for Nodule Autoregulation Receptor Kinase *NARK*) which are a non-nodulation and supernodulation mutants, respectively, as a result the expression of legume-specific miR1507, miR1511 and miR1512 was compromised in both mutants [96]. Another interesting and apparently specific symbiotic miRNA is miR5229a/g, which was identified in mycorrhizal roots of *M. truncatula* plants infected with *Glomus intraradices* [84]. MiR5229a/g which is the most strongly induced, was found by in situ hybridization to be exclusively expressed in arbuscules-containing cells in the root cortex, albeit with different signal intensities indicative of a specific function during different stages of the arbuscule development [84]. miR167 was localized in the differentiating peripheral vascular bundles and the novel miRNAs miR2586 and Mtr-s107 accumulate in the nodule meristem, leading the authors to conclude that miRNAs accumulate mainly in undifferentiated cells [94].

As stated before, rhizobacteria and AM share several elements of their symbiosis pathway [6] and miRNAs-mediated regulation is not an exception. miR169 which targets the CCAAT-binding transcription factor MtHAP2-1, was identified in the symbiotic interaction occurring between *Glycine max* and *B. japonicum* [83]. This transcription factor is highly induced during symbiosis and its degradation is mediated by miR169, causing a delayed nodule development and subsequent inability to fix N<sub>2</sub> [101]. This miRNA was also found to be up-regulated in *Medicago* interacting with AM, accumulating in the phloem and around fungal hyphae [84]. Another crosstalk must be established between symbiosis and nutrition pathways to determinate if the colonization of microbes occurs or not. An illustration of this comes from miR167 and miR5204 which are up-regulated in *Medicago* mycorrhizal roots under low phosphate conditions, as compared to nonmycorrhizal roots [84]. However these miRNAs were also regulated by phosphate, pointing to a direct connection between nutrition and symbiosis. Previous studies demonstrated an induction of miR399 under Pi depleted conditions in mycorrhizal *M. truncatula* and tobacco plants associated with a concomitant increase in Pi content [102]

## 12. Conclusions and perspectives

Research in sncRNAs is ultimately one of the most active and promising fields in plant biology, and it is expected to grow even more in importance in the near future, however many aspects of sncRNAs functions during plant-microbe interactions still remain unclear. How are these sncRNAs regulated? Are there common regulatory and feedback regulatory circuits between the different classes of sncRNAs? Are there core sncRNAs and targets for different class of pathogens and for different plant species? What is the evolutionary history of these different families of sncRNAs and how did they shape plant evolution? How did differences in sncRNA regulation across the plant kingdom arise? How are new sncRNA-specificities generated and how variable can these molecules be within species or populations? Studies reviewed here highlight the importance of sncRNAs in gene regulation in response of plants to pathogens as diverse as viruses, bacteria and fungi. Some sncRNAs have been shown to be induced or repressed in response to these diverse pathogens during incompatible and compatible interactions indicating a dual role of these RNAs as positive and negative regulators of plant immunity. This fact demonstrates the complex network of gene expression during plant-microbe interactions and should be considered in biotechnological programs focused to enhance the crop resistance to plant diseases. The notable repression of *R* genes during symbiotic interactions stresses the importance of these molecules during plant-microbe interactions and provides a bridge between pathogenic and beneficial interactions. The role that effectors have and its interaction with the plant silencing machinery reveals also the amazing and surprising mechanism that pathogens have evolved to surpass the plant immunity mechanisms. Deepening on all this knowledge surely will open new ways to improve resistance against biotic stress in several plants including crops of economical importance.

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