RNA silencing in plant immunity: beyond the arms race?

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Abstract

RNA silencing is well established as an anti-viral system in plants in which small(s) RNAs guide Argonaute protein effectors of defense to targets in the viral RNA or DNA. Virus-encoded suppressors of silencing counteract this defense system. This review summarises recent findings about anti-viral RNA silencing including the movement of RNA through plasmodesmata and how the plant differentiates self *vs* viral RNAs. We also describe the emerging picture that, beyond antiviral defense, RNA silencing has a role in plant immunity against non-viral pathogens. This effect on general immunity is mediated by trans-kingdom movement of RNA into and out of the infected plant cells through vesicles and other structures and through the action of silencing suppressors encoded by these organisms. There is also an effect of RNA silencing on general immunity because host-encoded sRNAs, including micro(mi)RNAs, regulate Nod-like receptors and defense signalling pathways in the innate immune system of plants. These RNA silencing pathways form a network of processes with both positive and negative effects on the immune status of plants.

Introduction

RNA silencing in plants was first identified as a post transcriptional mechanism in transgenesis and virus-infection[1,2]. It is triggered by viral or transgene RNA and key intermediate molecules are double stranded (ds) or hairpin RNA substrates of Dicer (DCL in plants). In some systems the dsRNA is generated by an RNA-dependent RNA polymerase (RDR) acting on a single stranded molecule and the 21-24nt RNA DCL-derived fragments are known generically as small (s)RNAs (Box 1). Single stranded derivatives of these fragments form nucleoproteins with Argonaute (AGO) proteins and they guide them by Watson-Crick base pairing to target RNAs. AGOs are nucleases and, in canonical RNA silencing (Figure 1a), the target RNA is cleaved opposite position 10 of the sRNA although there are variant mechanisms as described below.

This system is effective in antiviral defense because specificity is conferred by the sRNA derived from the viral genome. It also has an amplification property due to the Dicer cleavage of each double stranded RNA into multiple sRNAs (Box 1). Additionally, the sRNAs are mobile between cells so that they could move with or ahead of the viral RNA and prime AGOs at or ahead of the infection front (Figure 1b). With RNA silencing, as with other defense systems and consistent with the 'arms race' concept of host-pathogen interactions, viruses encoded suppressors that counteract the defense role of RNA silencing [3–5] (Box 2 and Figure 2).

Animals including worms, insects and mammals produce viral sRNAs in infected cells[2,6,7] and it is likely that protection against viruses is a conserved and ancient role of RNA

silencing. During eukaryotic evolution, however, there has been mechanistic and functional diversification of RNA silencing that is especially pronounced in plants[8,9]. Variations on the basic RDR/DCL/AGO pathway have diverse defense-related roles so that, as described in this review, the role of sRNAs in immunity is much more extensive than simply as an antiviral defense system. It is associated, for example, with cellular pathogens and symbionts in addition to viruses and it is part of the regulatory networks associated with multiple layers of the plant immune systems.

In this review we first discuss outstanding questions about RNA silencing and anti-viral defense including the ways that the plant's silencing machinery differentiates viral and host RNAs: a question of self *versus* non-self. Other virus-defense related issues include the movement of sRNA in the infected plant and the possible targeting of host RNAs by virus-derived sRNA. We then discuss RNA silencing in the context of cellular pathogens with emphasis on the two-way traffic of host- and cellular pathogen-derived sRNAs, the possible mechanisms of trans-kingdom RNA transport and its role in virulence and defense. Finally, we discuss the role of RNA silencing in the regulation of immunity and how this regulation is perturbed by infection. Our conclusion is that RNA silencing provides a regulatory network in which the multiple immune systems can be fine-tuned. This network requires a more nuanced perspective on host pathogen interactions than the 'arms race' in which the host's immune systems prevent virulence of the pathogen and the pathogens overcome these defenses[10].

Anti-viral RNA silencing pathways

The proliferation of RNA silencing pathways is associated with multi-gene families and consequent functional diversification of the various RDR, DCL and AGO proteins. Associated with these different pathways there are variant species of sRNA and an unnecessarily complicated nomenclature (Box 1). One of the major types – siRNAs - are produced by DCL cleavage at multiple sites in a perfectly double stranded precursor RNA. The miRNAs, in contrast, are produced by DCL cleavage at two sites in an imperfectly base-paired long RNA: each precursor gives rise to a predominant single species. Even the distinction of siRNA and miRNA, however, is not clear cut because miRNA loci have an evolutionary origin as siRNA loci and there may be transition types of sRNA that are not easily classified[11]. Within the siRNA category there are many variants (Box 1) including tasiRNAs, phasiRNAs, P4-sRNAs, easiRNAs, nat-siRNAs as well as subclasses of 21nt, 22nt or 24nt length. The classification of these variants is often based on secondary characteristics and the categories may overlap. To avoid confusion, in this review, we refer to miRNAs where appropriate but for other categories we use the generic term 'sRNA'.

In this section of the review we describe how understanding of the diverse RNA silencing pathways gives a better view of defense against different types of virus and how the process can be specific for viral rather than most host RNA. We also assess the movement of viral sRNAs between cells and the potential of viral sRNAs to target host genes.

- ***RNA silencing and posttranscriptional suppression of viruses.***

In *Arabidopsis* the diversification of RNA silencing is indicated by the DCL1-4 proteins producing, respectively 21,22, 24 and 21nt sRNAs that are each associated with distinct RNA silencing pathways of which three are primarily cytoplasmic and one is primarily nuclear[12]. The DCL2 isoform generates 22nt sRNAs for which the targeted RNA is repressed at the translational level[13] or, rather than being cleaved as with 21nt sRNAs, it is converted into a dsRNA by an RDR[14,15] (Figure 1a and Box 1). This dsRNA would then be the substrate of DCL1 or DCL4 and the precursor of multiple secondary sRNAs.

In the context of antiviral defense this secondary sRNA system has the potential to form a cascade of silencing RNA in which primary sRNAs prime the synthesis of multiple secondary sRNAs[16]. This cascade would add to the amplification property of the canonical RNA silencing pathway in an infected plant and the suppression of viral RNA translation by 22nt sRNAs would also reinforce the anti-viral potential of this DCL2 pathway (Figure 1a).

The 22nt sRNA is more likely to promote secondary sRNA more efficiently than 21nt species because the additional nucleotide at the 3′ end protrudes from the AGO nucleoprotein structure and binds to the SGS3 protein[17]. This AGO–SGS3 complex causes ribosome translocation on a mRNA to stall and it allows recruitment of the RDR. The protruding 3′ end of the 22nt sRNA would then be available as a primer for the dsRNA production by the RDR. DCL2 generates 22nt sRNAs and it features prominently in antiviral RNA silencing[18–21].

Other diversification of posttranscriptional RNA silencing is associated with anti-viral defense but it has less obvious functional implications than with the DCL2 pathway. The DCL1 and DCL4 isoforms, for example, are both associated with 21nt viral sRNAs[20–22]. Similarly, at least six of the ten AGO isoforms have antiviral roles[23] and RDR1 and RDR6 both suppress virus accumulation exemplified in many systems[24] (Figure 1c). The possible relevance of this pathway diversity in the context of virus defense is discussed below in the section on "RNA silencing of viral rather than host RNA".

- ***RNA silencing pathways and transcriptional silencing of viral DNA.***

An important variation on RNA silencing operates in the nucleus and it protects against transposons and DNA viruses[25,26]. The RNA substrate for this nuclear pathway is transcribed by a variant form of DNA-dependent RNA polymerase II (PolII) known as PolIV. PolII and PolIV are multi-subunit proteins and they share some of the smaller subunits. The RDR2 and DCL3 isoforms carry out similar functions as their homologues in the posttranscriptional pathways described above but the sRNAs in this pathway are 24nt rather than 21nt or 22nt. PolIV might also produce the sRNA directly without DCL cleavage[27,28]. The 24nt sRNA effector complex has an AGO nucleoprotein (AGO4) as in the canonical pathway but its role, rather than RNA cleavage, is to recruit DNA methyl transferase DRM1/2 and to methylate DNA cytosines at the target site. Targeting involves nascent, chromatin-associated transcripts produced by PolV - a second variant of Pol II in which the largest subunits are distinct but with smaller subunits common to PolII or PolIV[29,30]. The chromatin-associated transcripts are a scaffold for targeting of the sRNA and AGO4 complex and the resulting methylated DNA is normally associated with transcriptional gene silencing (TGS). The overall process is known as RNA-directed DNA methylation (RdDM)[25,26,31] (Figure 1d).

Integral to the RdDM pathway there are feedback systems ensuring stable maintenance of the silent state. SU(VAR)3–9 like histone methyltransferases2/9 (SUVH2/9) proteins are key components of the feedback because they bind the methylated DNA and recruit PolV[32–34]. Other feedback systems involve SUVH proteins that associate with the methylated DNA and introduce repressive histone modifications to the nearby nucleosomes. The SAWADEE HOMEODOMAIN HOMOLOGUE 1 (SHH1) protein binds to the modified histones and it recruits PolIV (Figure 1d) to reinforce the RdDM. This RdDM pathway has been elucidated in the context of transgenes and transposons but it is also involved in defense against DNA viruses or virus-like transposons. It is likely for example that RdDM is associated with silencing of caulimoviral[35] and geminiviral DNA[36,37]. Retrotransposons are similar in many respects to retroviruses and they are also silenced by RdDM[26].

- ***Suppressors of RNA silencing.***

Viral suppressors of RNA silencing (VSRs) may sequester the sRNA away from an AGO, prevent access to dsRNA by Dicer, mediate degradation of proteins in the RNA silencing pathway or affect movement of an RNA silencing signal in the plasmodesmatal connections between cells[38,39]. Recent reviews describe current understanding of viral suppressors of silencing[3–5] and, in Box 2 and Figure 2, we summarise the VSRs referred to in this review and their mode of action.

There are also host-encoded suppressors of silencing of which rgsCam is the best characterised. It is a calmodulin-like[40] protein and, in infected plants, it interacts with and enhances the VSRs of tobacco etch virus HC-Pro, ßC1 of tomato yellow leaf curl China virus[41] and tomato golden mosaic virus AL2[42]. A second class of host-encoded suppressors are RNase III-like proteins (RTL1) that are induced in virus-infected plant[43]. RTL1 proteins degrade the dsRNA substrate of DCL proteins and thereby inhibit viral sRNA biogenesis.

- ***RNA silencing of viral rather than host RNA.***

The replication intermediates of RNA viruses are dsRNA that, in principle, could be the DCL substrate in RNA silencing. It is likely, however, that viral single stranded RNAs are also sources of sRNA in plants through various mechanisms including those involving RDRs. Endogenous host mRNAs in contrast are not templates for sRNA unless they are targeted by miRNAs or 22nt sRNAs that trigger secondary sRNA production[14,15] (Box 1, Figure 1a). This difference of host and viral RNA implies differentiation of non-self (viral or transgene) vs self (host) RNAs.

In part the lack of 'self' or host mRNA silencing is likely because proteins bound to 5' cap (Cap binding proteins) or 3' poly A structures and proteins associated (Poly-A binding proteins) inhibit access by RDR, DCL or other factors involved in sRNA biogenesis[44] (Figure 3a). Viral RNAs, however, may similarly have these terminal features and there must be other factors that override any block on sRNA biogenesis of these non-self RNAs, such as the presence of unprotected secondary structures or the presence of dsRNA replication intermediates (Figure 3b). Another plausible scenario invokes subcellular compartments for viral RNAs that can be accessed by isoforms of proteins involved in sRNA biogenesis. These compartments may be related to replication

factories or 'viroplasm' (Figure 3c) in virus-infected plants that are associated with viral RNAs or the virus-encoded replication and movement proteins[45]. Host-encoded proteins in the endomembrane and cytoskeletal systems may also be present.

Relevant to this compartmentalization hypothesis, there is evidence for specific subcellular localization of DCL, AGO and other proteins in RNA silencing pathways[46–48]. Unfortunately, these studies do not extend to virus-infected plants and, to test the importance of compartmentalization, we need more information about the locations of isoforms of RNA silencing proteins (Figure 3c) relative to the viroplasms or features of infected cells. DCL2 in tomato, for example provides more protection against tobacco mosaic virus (TMV) than with potato virus X (PVX)[18] and may be more strongly located with the TMV rather than PVX replication factories or viroplasms. Similarly there are other differences between viruses that should correspond to colocalization with the various types of replication factory or viroplasm. AGO2 silences turnip crinkle virus (TCV) but not TMV in *Arabidopsis* and AGO2/AGO5 targets PVX in *Arabidopsis*[49]. AGO requirements for anti-viral RNA silencing may also vary between cell types[23,50].

In addition to the compartmentalization of viral RNAs as an explanation of non-self silencing, there may also be a quantitative threshold. This threshold hypothesis was originally invoked to explain silencing of transgenes[51] but it could also apply in virus infection. Viral or transgene RNA silencing would start when viral or transgene transcripts increase above a certain level so that they saturate the cap or polyA binding proteins that are thought to prevent sRNA biogenesis[44] on host mRNAs (Figure 3d). The excess molecules would then be available for RNA silencing.

Competition between DCL isoforms for sRNA precursors might also influence a threshold. The DCL1 and 4 isoforms, for example, compete with DCL2 for sRNA precursors[21]. Early in an infection cycle when viral RNA levels are low we envision that low DCL1 and DCL4 would prevail and produce limited RNA silencing. As the level of viral RNA increases, however, these 21nt DCLs would be saturated so that DCL2 could produce 22nt viral sRNAs. These sRNAs in turn would trigger multiple rounds of secondary sRNA production and rapid amplification of the anti-viral RNA silencing system (Box 1 and Figure 3e).

A similar threshold process in RdDM of DNA viruses is illustrated by the progressive silencing of a virus-like retrotransposon – Evadé - in Arabidopsis. The DNA of this retrotransposon is transcribed by Pol II and 21nt or 22nt sRNAs are produced by DCL4 and DCL2[26] (Figure 3f). At this stage, equivalent to the early stage in a DNA viral infection cycle, there would be posttranscriptional silencing targeted to viral RNAs. After several generations when the Evadé RNA accumulates to a high level, however, the posttranscriptional pathways would be saturated and there is transition to the RdDM pathway and transcriptional gene silencing, as in the later stages of a DNA viral infection cycle (Figure 3f). Consistent with this threshold hypothesis, a loss of function in *DCL2*/*DCL4* enhances RNA virus-induced RdDM of nuclear DNA[52].

Other steps in the establishment of RdDM on viral DNA are indicated by a recent study with a retrotransposon transgene. It demonstrates that initial recruitment of an AGO nucleoprotein to the target DNA is independent of PolV[53] and there could be PolII transcripts that have the scaffold function of the PolV RNA in the canonical RdDM

pathway[54] (Figure 3g). Alternatively, a direct AGO-sRNA-DNA interaction forming an R loop has not been conclusively ruled out (Figure 3g).

- ***Mobile sRNA in virus defense***

Viral and other sRNAs move between cells and through the vascular system so that, in principle, RNA silencing from the initially infected cells would move with or ahead of the virus and influence its accumulation elsewhere in the plant[55] (Figure 1b). However, the extent to which viral sRNA movement influences spread of viruses in plants is likely to vary from plant to plant and virus to virus. In PVX-infected *Nicotiana benthamiana*, for example, the mobile RNA might prevent virus accumulation in the apical regions from which the virus is normally excluded[56]. Similarly, with a mutant tombusvirus, there are *in situ* hybridization data indicating that viral sRNA could restrict movement out of the phloem[57]. In oil seed rape mosaic virus-infected *Arabidopsis*, the mobile viral sRNAs may prevent expression of viral proteins and symptoms but not the accumulation of viral RNA indicating suppression of translation[58].

In none of these systems, however, is there direct evidence for viral sRNA movement. To resolve the proposed involvement of mobile sRNA in virus disease it would be helpful to know more about the process of RNA movement including the molecular form of the mobile RNA and the channels through which it moves. Various molecules including long single-stranded RNA, dsRNA, free/AGO-bound secondary sRNA or primary sRNA have been candidates for the mobile silencing RNA and a recent analysis implicates free sRNA duplexes[59].

This evidence is based on sequestration of the silencing signal *en route* by a tomato bushy stunt virus (TBSV) VSR - P19 – that binds to sRNA duplexes (Figure 2). AGOs bind preferentially to sRNA depending on the 5' nucleotide and, at distal sites, the mobile sRNAs were depleted for those with 5' A and U (preferred by AGO2 and AGO1 respectively). Those with 5'G or 5'C were not depleted because there are no AGOs specific for 5'G sRNAs or because AGO5 that binds sRNA with 5'C is not expressed inthe tissue analysed. The finding that DCLs are required for mobile silencing in incipient rather than recipient cells is also consistent with sRNA as the mobile molecule[59] (Figure 1b). These data are relevant to movement between adjacent cells but the promoter used to express the P19 'interceptor' is not expressed in phloem[60] and so further analysis of long distance movement is required.

In other studies there is also ambiguity about the size of mobile sRNA. Some evidence indicates that 22nt sRNA products of DCL2 are strongly associated with mobile sRNA silencing, for example, between host plants and *Cuscuta campestris* parasitic plants[58,61] but this interpretation may be influenced by amplification of secondary sRNA production. Other evidence indicates that all size classes of sRNA are potentially mobile (reviewed in[62]).

At the cellular level the current understanding of the movement channels of mobile sRNA invokes plasmodesmata[62] that create a symplastic connection between cells and into the phloem of the plant. The patterns of silencing in plants are consistent with the involvement of plasmodesmata because there is exclusion of mobile silencing from symplastically isolated cells[63]. Depending on the source of silencing RNA and the plant species, there can be movement of the silencing signal following source–sink

relationships via the phloem or in the opposite direction and between parenchymal cells. That the tomato yellow leaf curl virus (TYLCV) C4 and TBSV P19 VSRs (Box2) target plasmodesmatal membrane bound kinases (BAM1 and BAM2)[38,39] (Figure 2) is also consistent with symplastic movement of sRNA as is the effect of gain of function mutation of callose synthase[64]. Callose lines the sleeve of the plasmodesmata and manipulation of its production could reduce the size exclusion and serve as a useful tool to investigate sRNA movement[65].

There are, however, large gaps in our understanding of sRNA movement. Mutant screens have been uninformative about plasmodesmatal involvement (reviewed in [66]) and we do not fully understand the full extent to which viral sRNA is a mobile immunity factor affecting the progression of virus disease. The recent finding that sRNA moves as a duplex before it associates with AGO in the recipient cell[59] may make it possible, finally, to gain this understanding.

- ***Viral sRNAs targeting host genes***

From the effects of experimentally modified viruses carrying fragments of host genes it is clear that viral sRNAs can target nuclear as well as viral RNAs and they can target methylation of nuclear DNA[52]. Correspondingly, there are examples of natural viral sRNAs from unmodified viruses targeting the transcripts of nuclear DNA. The Y satellite RNA of cucumber mosaic virus (CMV), for example, generates sRNAs that target a *CHL*1 host mRNA involved in chlorophyll biosynthesis[67,68] and a viroid-derived sRNA targets callose synthase – a defense-related enzyme (see below)[69].

There is also evidence that viral sRNAs trigger secondary sRNAs using host mRNAs as RDR1 templates[70]. Similarly, in turnip mosaic virus (TuMV)-infected rapeseed, the viral sRNAs trigger secondary sRNA production using the targeted host RNAs as a template including the autophagy cofactor *NBR*1 mRNA[71]. In principle there also is the potential for viral RdDM. In an artificial system with a transgene target this viral RdDM can be induced even if there are up to four mismatches with the target DNA[72].

The virus-induced host gene silencing may cause symptoms. The Y satellite-targeted *CHL*1 mRNA, for example, causes a yellow mosaic symptom on infected tobacco[67,68]. TYLCV-induced silencing of a long noncoding RNA SlLNR1 similarly causes stunting and leaf curling associated with the associated disease[73]. With TuMV-induced silencing of *NBR*1 mRNA, the host gene silencing may be associated with reduced immunity in the infected plant[70].

One interpretation of these viral sRNA interactions is that they increase viral fitness and they reflect adaptation of a virus to its hosts. The CMV Y satellite-induced yellowing is likely, for example, to attract aphids to the infected plant[74] and thereby increase the aphid transmission of the virus between plants. Similarly viral fitness may be enhanced by suppression of immunity due to RNA silencing in the infected plant[70]. The experimental evidence to support this hypothesis, however, is often lacking. There should be evidence of specific co-adaptation or experimental manipulation of viral sRNA interaction to test the predicted effect on the virus or on its spread between plants. This need to validate the biological relevance of the pathogen sRNA-host target RNA interaction is relevant also, as discussed below, in the context of cellular pathogens.

RNA silencing and cellular pathogens

RNA interference –RNA silencing triggered by exogenous dsRNA – requires direct delivery of dsRNA into the cell from a transgene or the uptake of environmental RNA into cell. The evidence for direct uptake of silencing RNA was first established in worms[75] and it was not surprising, therefore, that transgenic dsRNA can transfer from plant cells into parasitic nematodes during their feeding cycle (reviewed in[76]). More surprising, however, are the emerging findings that RNA silencing transfers between cells in other contexts: plant-fungus, plant-oomycete, plant-bacterium and between hosts and parasitic plants[61,77]. There is the potential, therefore, for two-way RNA silencing traffic to influence the interactions of plants with other cellular pathogens and symbionts as well as with viruses.

- ***Trans-kingdom RNA silencing between plants and fungi or bacteria.***

The first indication of RNA traffic from plants to their infecting fungi was from 'host induced gene silencing' (HIGS): experimental host RNA targeted to essential fungal genes resulted in resistance and reduced fungal growth. First demonstrated in pathogenic fungus *Blumeria graminis*[78,79] these findings are now confirmed in several fungal pathogens including *Puccinia striiformis*[80], *Fusarium verticillioides*[81], *Puccinia triticina*[82], *Fusarium oxysporum* f. sp. *cubense*[83], *Sclerotinia sclerotiorum*[84], *Botrytis cinerea*[85], *Fusarium culmorum*[86] and the oomycetes *Bremia lactucae*[87], *Phytophthora infestans*[88] and *Phytophthora capsici*[89]. There is also HIGS involving a mutualistic symbiosis between *Medicago truncatula* and a mycorrhizal fungus[90] and trans-kingdom RNA silencing between *Arabidopsis* and the Gram-negative bacterial pathogen *Pseudomonas syringae* DC3000, as reported in a preprint[91]. Precisely how the transported RNA would affect gene expression in the recipient cell remains to be determined. It is not likely to be through the canonical silencing pathway described above, however, because the necessary proteins are strictly eukaryotic.

It is likely that HIGS is based on a defense system in which host miRNAs and sRNAs mobilize into the fungal cell. Mutant *Arabidopsis* that was defective for AGO1, RNA helicases RH11 and RH37 and RNA binding annexins 1 and 2 secreted sRNAs less efficiently into *B. cinerea* than the wild type[92]. These mobile sRNA included tasiRNAs (Box 1) and their predicted targets in the fungus include virulence factors such as vacuolar protein sorting 5a large subunit of the dynactin complex and a suppressor of actin (SAC1)–like phosphoinositide phosphatase that regulates secretory membrane trafficking[93]. There may be a similar transfer of tasiRNAs (Box 2) from *Arabidopsis* into the oomycete *Phytophthora capsici*[89] and of miRNA from cotton into *Verticillium dahliae*[94]. In both systems the proposed targets include virulence factor mRNAs and, consistent with that hypothesis, *V. dahliae*[94] was hypervirulent if the trans-kingdom miRNA was destabilized in the plant, or if the target mRNAs in the fungus had mutations in the miRNA target site[94].

That cellular pathogens encode suppressors of RNA silencing is consistent with HIGS as a defense system. These suppressors, known in a plant pathogenic bacterium[95], oomycetes[89,96–99] and a fungus[100], could be virulence cofactors because they are transported into the host and, parallel to the role of VSRs (Figure 2), they could suppress the trans-kingdom silencing of virulence-related mRNAs in the pathogen.

Trans-kingdom RNA silencing in the reverse direction from cellular pathogens into plant cells may also influence disease. With fungi, for example, there are *B. cinerea* sRNAs delivered into infected cells of *Arabidopsis* where they are incorporated into AGO nucleoproteins and target mRNAs required for plant immunity[101]. *Puccinia striiformis f. sp tritici*[102] and *Fusarium graminearum* infections may also involve sRNA transfer into infected plant cells[103]. Similarly the oomycetes *Hyaloperonospora arabidopsidis* and *Phytophthora infestans* also transfer sRNA into infected plants where they target mRNAs contributing to plant immunity[104,105]. The *H. arabidopsidis* sRNA transfer was validated because the oomycete sRNAs were incorporated into plant AGO1 nucleoproteins.

A key functional test with *P. infestans* on potato involved target mimic RNA of an oomycete miRNA that is transported from *P. infestans* into infected potato. The putative plant mRNA target encodes a critical defense component and, consistent with the role of the trans-kingdom miRNA as a virulence factor, the target mimic strains of *P. infestans* were less virulent than the control strains[105].

The trans-kingdom sRNA transfer is not restricted to pathogens. In a mutualistic bacterial infection, there are tRNA fragments transferred from *Sinorhizobium meliloti* into the recipient soybean root cells that are destined to form root nodules[106]. The transferred RNAs form AGO1 nucleoproteins in the recipient cell and the targets are conserved sites in negative regulators of root nodulation mRNAs. The encoded proteins of these targets including *ROOT HAIR DEFECTIVE 3* (*RHD3*), *HAIRY MERISTEM 4* (*HAM4*), and *LEUCINE-RICH REPEAT EXTENSIN-LIKE 5* (*LRX5*), are directly involved in root hair development – an important stage in the early establishment of the nitrogen fixing root nodule symbiosis[107]. In a mutualistic mycorrhizal fungal interaction with *Eucalyptus grandis*, a novel fungal miRNA is transported to roots. It enhances the mutualistic interaction, by target targeting defense mRNAs including some that encode Nod-Like Receptors (NLRs) required for the plant's immune system[108].

- ***Transport of trans-kingdom sRNA***

The transport mechanism of trans-kingdom RNA silencing cannot be the same as the sRNA transport between parasitic plants and their hosts or within plants because there are no plasmodesmata at the interface of plants and fungi or bacteria. There must be a separate mechanism of trans-kingdom silencing in which RNA crosses the membranes and cell walls of both organisms[109].

One candidate mechanism for the plant to symbiont transfer involves tetraspanin exosome-like extracellular vesicles (EVs). They could capture the silencing RNA in the cytoplasm of the plant cell and secreted it into the fungal cell possibly by fusion with the plasmalemma of the fungal cell. There is good evidence that these vesicles fuse with the fungal membrane and they clearly play a role in plant defense: tetraspanin mutants (*tet*8, *tet*9) are hypersusceptible to fungal infection. The HIGS sRNA is resistant to nuclease treatment but not to combined detergent and nuclease[93] consistent with it being associated with these vesicle. However a more stringent test of intra-vesicular status is resistance to nuclease and protease treatment[110]. Recent evidence based on this stringent test indicates that HIGS RNA is extra-vesicular, is enriched for m^6-methyladenine modification (m^6A) and is in complexes with a glycine rich m^6A RNA binding protein and AGO2[111] (Figure 4a). At this stage we should keep an open mind

about the possibility of multiple transport mechanisms and avoid the pitfalls that have complicated research into mammalian vesicle transport[110].

In the reverse direction the transfer from fungus to plant is also not well understood. One possibility, if not within or associated with vesicles, involves membrane tubules like those adjacent to hyphae of a mycorrhizal mutualist making contact with plant cells[112]. Alternatively there is a complex of seven infection-specific proteins of *Ustilago maydis* that is anchored in the fungal membrane. This complex protrudes into host plant cells and is likely to make contact with channel-forming plant plasma membrane proteins. The complex is thought to mediate transfer of effectors between the fungus and the host plant[113] (Figure 4b) and, although *U. maydis* does not have the canonical sRNA machinery, similar channels could be involved in other silencing-competent species including *U. hordei* [114].

A further possibility, for transport in either direction, is suggested by the recent reports that RNA applied to plants confers specific protection against cellular and viral pathogens [115,116]. Presumably this RNA crosses cell walls either as naked RNA or, more likely, in association with extracellular proteins (Figure 4).

- ***Biological and evolutionary consequences of trans-kingdom RNA silencing***

Although the process of trans-kingdom RNA transport is well supported by evidence, there are gaps in the available data and outstanding questions about its biology. Of the gaps, the most pressing, in some systems, is a direct functional assay of the transferred RNA in the recipient cell. It would be appropriate, for example, to test the function of trans-kingdom RNA by mutation of the target site or ectopic expression of genes with target site mutations[94]. Target site mimic RNAs of the trans-kingdom sRNA are also useful although ideally they should be expressed in the recipient rather than the incipient cell[94,105]

There is also gap in the evolutionary analysis of trans-kingdom sRNA that should show evidence of co-evolution of the host and the symbiont as with the trans-species sRNAs from the wide host-range parasite plant *C. campestris*. These sRNAs target conserved regions in the host plant mRNAs and there is variation of the simultaneously expressed sRNAs corresponding to synonymous sites in the target RNAs[117]. This compensatory sequence variation ensures that the sRNAs find targets in the wide host range of *C. campestris*. In the *S. meliloti* soybean system the trans-kingdom sRNA targets correspond to conserved regions in the mRNA of negative regulators of root nodulation[106,107] consistent with a co-evolutionary adaptation in this mutualistic symbiosis. However, rigorous testing of this model requires data on target site conservation in plants that do not nodulate[118]. Correspondingly, the host RNA targets of trans-kingdom sRNAs from broad host-range pathogens should be more highly conserved than those of specialized pathogens.

One co-evolution hypothesis related to the *Arabidopsis-P. capsici* pathosystem is that trans-kingdom sRNAs from the plant are like a shotgun targeted randomly to virulence genes in the pathogen[77]. Even in that scenario, however, there would be a sequence footprint because there is a low probability that any of the $>10^{12}$ possible 21mers would correspond to targets in the pathogen's virulence genes. If these trans-kingdom sRNAs

do have a role it is likely that there would have been some enrichment by selection: the shotgun would not have been completely random. The evidence for a shotgun strategy should also be evident from bioinformatic analysis because the trans-kingdom sRNAs should target 'non-self' RNA sequences in the pathogen in preference to 'self' sequences in plant.

RNA silencing in a network of immune systems.

RNA silencing is one of many layers of defense providing protection against diverse pests and pathogens. There are, for example, physical or chemical barriers including cutin coatings[119], thickening of cell walls[120] or preformed antimicrobial compounds[121] that prevent the initial colonization by diverse pests and pathogens or the vectors of viral disease (Figure 5). There are also inducible immune systems in which host-encoded receptors trigger signal transduction pathways in the presence of the pathogen and biochemical or molecular changes that prevent disease (Figure 5). In some examples an induced cell death – the hypersensitive response – is associated with inducible defense.

These inducible systems involve both cell surface and intracellular receptors[122,123]. The cell surface receptors known as pattern recognition receptors (PRRs) bind to conserved, essential features of the pathogen (microbe- or pathogen-associated molecular patterns - MAMPs or PAMPs) including chitin in fungi or flagellar proteins in bacteria. The receptors are receptor-like kinases (RLKs) in the plasma-membrane and PAMP recognition triggers MAP kinase mediated signal transduction pathways in the infected cells. This layer of defense is referred to as PAMP-triggered immunity (PTI). The intracellular receptors, in contrast, are Nod-like receptors (NLRs) that recognize virulence effectors from the pathogen and they account for effector-triggered immunity (ETI) (Figure 5). Both ETI and PIT are associated with downstream processes involving salicylic acid – a defense hormone – and the activation of defense gene expression (Figure 6a).

The defense barriers, PTI, ETI and RNA silencing are often represented as independent components of the plant's immune systems. An emerging picture, however, is that they are linked. The activation of ETI, for example, enhances PTI-induced defense systems including reactive oxygen species and defense gene activation[124,125] and *vice versa*[126]. There is also link with barrier defense systems because they are damaged by hydrolases and other pathogen-derived effectors to release Damage-associated Molecular Patterns (DAMP) that bind to PRR-like receptor kinases and, consequently, trigger PTI[127] (Figure 5).

In this section we review the interactions of RNA silencing with ETI and PTI immune systems of plants and how these interactions can either reduce or reinforce virulence and defense. Most of the examples involve viruses but, given the emerging findings of trans-kingdom RNA silencing and silencing suppressors in cellular pathogens, it is likely that there will be similar connections in many types of plant disease. The following discussion includes reference to the negative regulation of ETI and PTI by miRNAs (Box 1), by other repressors of immunity and by RdDM of defense genes (Figure 6a). We refer in this discussion to virulence as a quantitative property of the pathogen associated with its accumulation and damage to the host[128].

- ***sRNAs and suppressors of silencing reinforce virulence by targeting multiple layers of defense***

The trans-kingdom or viral sRNAs that silence defense-related mRNAs in the host are one illustration of how RNA silencing interacts with other immune systems[101–105]: the targeted mRNAs in the plant cell are often associated with ETI, PTI or other defense systems (Figure 6b). The suppressors of RNA silencing might also block defense because they bind to AGO4 and PolV (Box 2) that may have a role in sRNA-independent defense systems[129,130] as well as in RNA silencing.

VSRs (Figures 2 and 6b) are also involved in interactions with sRNA-independent defense systems because they are multifunctional proteins. The CMV (2b)[131,132] and cauliflower mosaic virus (P6)[133] VSRs for example, additionally to silencing suppression (Box 2), block the effects of salicylic acid – a defense hormone that acts in the downstream ETI and PTI pathways[132]. The cotton leaf curl Multan geminivirus V2 geminiviral suppresses silencing by interfering with the RDR6/SGS3 pathway of antiviral silencing[134] and it blocks a calmodulin-binding transcription activator-3 (CAMTA3). CAMTA3 activates defense genes including a bifunctional nuclease when insect vectors of the virus feed on plants[134] (Figures 6b).

The possibility of multifunctionality has not been explored with most of the silencing suppressors from cellular pathogens[89,95–100]. The AvrPto suppressor of RNA silencing in *P. syringae*[95], however, also prevents the binding of a PAMP receptors to accessory kinases when PTI is being triggered[135](Figure 6b). PSR2 is an effector of disease and suppressor of RNA silencing from *Phytophthora sojae*. It also likely to be multifunctional because it has a repeat structure that is shared with other effectors that are not known suppressors of silencing[136].

An additional effect of VSRs involves insect vectors that spread viruses between plants. The CMV 2b viral suppressor of silencing, for example, binds to jasmonate ZIM-domain proteins and thereby prevents their degradation[137]. The biological effect of this action is reduced expression of genes that are normally associated with deterrence of aphid vectors of the virus. The plant is, consequently, more attractive to aphid vectors after virus infection. The 2b protein may also increase pollinator attractiveness of CMV-infected plants and confer enhanced fitness on the virus-susceptible genotypes[138]. These effects on vectors or pollinators are not strictly virulence-related but they illustrate the point that the suppressor proteins influence the progression of disease through their multifunctionality.

- ***sRNAs and suppressors of silencing enhance multiple layers of defense and reduce virulence***

    - *Suppressors of silencing and posttranscriptional mechanisms*

Viral- and other pathogen-encoded suppressors of RNA were first identified as virulence factors – they suppress the defense-related RNA silencing pathways and thereby enhance the extent to which the pest or pathogen can accumulate in the infected plant (Box 2). However, they may also confer avirulence. Some VSRs, for example, including P25 of PVX[139,140], NSs of tomato spotted wilt virus[141] and P38 of TCV[142] interact directly

or indirectly with NLRs and they trigger ETI: they are classical avirulence determinants (Box 2)(Figure 6c).

There are also miRNA-mediated processes through which silencing suppressors would activate immunity. These processes involve miRNA negative regulators of defense proteins and they are affected in infected plants because silencing suppressors (Box 2) target proteins and RNA intermediates that are common to both miRNA and sRNA pathways (Box 1). It is likely that, in infected plants, the miRNA-mediated negative regulation of immunity would be relieved by the pathogen-encoded suppressors of silencing.

Examples of these miRNA systems involve the miR472/482/2118 species that target a conserved P loop motif in the NLR mRNA[143,144]. The different members of this miRNA family (miR NLR) vary at positions corresponding to synonymous sites in this conserved motif so that a few miRNAs have the potential to target many different mRNAs in the NLR family[144]. Other miRNAs targeting NLRs include the miR9863 family that regulate a subset of barley *Mla* alleles for resistance against powdery mildew[145] and miR6019/6020 targeting *N* genes conferring resistance in tobacco against TMV[146] (Figure 6a, 6c).

It is striking that most of these miR NLRs are the 22nt length species that trigger secondary sRNAs on their targets[14,15,18,147]. In tomato there is also an additional non-coding RNA target of one of these miRNAs that has a mosaic structure due to rearrangement of multiple NLRs[148]. The likely consequence of this secondary sRNA production is that cells will have a large population of sRNAs targeted to many if not most members of the NLR gene families.

In some instances there is a developmental component to this miRNA-mediated silencing of NLR genes. The miR6019/6020-mediated suppression of the *N* NLR gene in tobacco decreased with age so that the level of resistance increased[149] and, in *Brassica napus*, there was a developmental increase in the level of miR1885 that correlated inversely with the level of its NLR target mRNA[150].

Other examples of viral defense-suppression by miRNAs include miR6026[18,19] and its DCL2 mRNA target, miR168 and AGO1 mRNA[151], miR162 and DCL2 mRNA[152] and miR403-mediated silencing of the AGO2 mRNA that is blocked by VSR P38 in TuMV-infected plants[153]. In these examples the defense mRNA target encodes proteins required for the biogenesis or RNA silencing activity of the miRNAs and there is a feedback characteristic to the negative regulation that would influence the dynamics of virus accumulation.

The biological consequences of miRNA-mediated silencing of defense systems are indicated by experiments in which target mimic RNAs in tomato blocked the action of miR482/2118b family members[148,154]. The target mimic plants exhibited enhanced basal resistance to *P. syringae* and *Phytophthora infestans*. There was a similar increase in basal immunity with a target mimic RNA of miR1885 in *Brassica napus*[150] and of defense against TMV but not PVX with target mimics of miR6026[18,19]. The target mimic RNA effect was not sufficient to block the disease caused by the various pathogens but it was sufficient to reduce their symptoms and accumulation in bioassays. A likely explanation of this result involves increased PTI mediated by enhanced level of the ETI-associated

NLR proteins[124,125]. It is likely that there are similar effects on basal immunity in infected plants due to the action of silencing suppressors on miR NLRs[144].

Similar to VSRs, the host-encoded suppressors of silencing could also contribute to the interactions between the layers of defense. These proteins may enhance viral virulence by interacting with VSRs, as discussed above[41,42], but they may also have an opposite effect. The rgsCam protein, for example, interacts with the dsRNA binding domain of the VSRs HC-Pro and 2b and triggers their degradation through the autophagy pathway[155]. Similarly the RTL1 suppressor of silencing that is induced in virus-infected plants[43] targets the dsRNA precursor of viral sRNAs. This activity would inhibit viral sRNA biogenesis but it would also block viral replication if its target is the dsRNA viral replication intermediate.

- *Pathogen-induced RNA silencing resulting in enhanced defense and reduced virulence*

RNA silencing is connected with general immunity because pathogen-induced sRNAs or miRNAs may target negative regulators of immunity including auxin signalling[156], a mitochondrial protein[157], a resistance associated protein[158] or a negative regulator of exocytosis[159]. Induction of these sRNAs or miRNAs in infected plants leads to an increase in resistance causing a decrease in pathogen virulence. Other miRNAs target resistance cofactors including superoxide dismutase[160] and other mRNAs[118](Figure 6c).

A different mechanism with a similar outcome occurs following rice stripe virus (RSV) infection that induces AGO18 through a process involving the the jasmonic acid defense hormone. The process also involves miR168 that targets the AGO1 effector of antiviral silencing[151]. AGO18 sequesters miR168 in an inactive form so that that level of the antiviral AGO1 increases[161,162]: immunity is enhanced in the infected plant.

- *Epigenetic mechanisms*

Other examples of defense gene activation in infected plants are associated with DNA hypomethylation and, in at least some of those examples, the effects are due to disruption of RdDM. There are striking changes to the epigenome, for example, in *Arabidopsis* treated with bacteria (either pathogenic or not), a bacterial flagellin-derived peptide, or the defense hormone salicylic acid. For both hypo- and hypermethylated DNA regions, there is association of these changes to the expression of adjacent genes[163,164]: Hypomethylated regions tend to be overexpressed whereas hypermethylated regions are associated with repressed transcript accumulation. Chemically-induced DNA hypomethylation enhances bacterial resistance in rice[165] providing direct evidence that these changes influence the immune status of plants. TuMV also influences gene expression via the epigenome and, in line with the findings that loss of epigenetic marks promotes defense, *Arabidopsis* mutants with a loss of epigenetic marks had enhanced tolerance of the virus relative to the wild type control[166] (Figure 6a).

The mechanisms accounting for pathogen-induced hypomethylation of defense genes may be independent of RNA silencing. WRKY and other defense genes, for example, acquired histone marks associated with active open chromatin (H3K4me3 and H3K9ac)[167,168]. Salicylic acid also promotes transcription of defense genes through

histone acetylation[169]. Other pathogen-induced genes, however, may be derepressed due to inactivation of proteins in the RdDM-mediated silencing perhaps through the action of suppressors of silencing of defense genes (Figure 6d) or induction of DNA demethylases[170] (Figure 6d). Up to 49% of the pathogenesis-related transcriptome, for example, is influenced by NRPE1- and ROS1-controlled DNA methylation following *Hyaloperonospora arabidopsidis*-infection of *Arabidopsis*[171].

A specific example of reduced RdDM leading to elevated defense is due to repression of AGO4 or AGO4a in *Arabidopsis* or a wheat relative after infection[164,172]. The elevated ROS1 DNA demethylase during PTI in Arabidopsis would have a similar effect because it results in the loss of RdDM in the promoter of a disease resistance gene *PMG1* or an immune receptor gene *RLP43*[170] (Figure 6d). In the mutualistic legume-*Rhizobium* nitrogen-fixing symbiosis a host encoded DNA demethylase DEMETER is required for nodule organogenesis[173]. A likely scenario is that it removes epigenetic repressive marks from genes involved in nodule development.

Infection-induced removal of repressive DNA methylation marks could have effects *in cis* on adjacent genes[170]. They could also influence gene expression *in trans* if the hypomethylated DNA produces sRNAs that could affect gene expression through any of the multiple RNA silencing pathways (Box 1). A likely scenario is that pathogen induced hypomethylation of transposon DNA leads to their transcription and sRNAs that silence other RNAs *in trans*. Consistent with this possibility in Arabidopsis tissues infected with Pseudomonas syringae pv. tomato there is hypomethylation and expression of pericentromeric TEs leading to sRNA production and RdDM. Also relevant is the finding that only 10-15% of genes with altered immune responsiveness in infected plants are adjacent to hypomethylated transposons or other sequence elements[170,171]: the hypomethylated elements could produce sRNAs acting *in trans*.

It is likely that epigenetic effects including removal of repressive marks on histones or DNA are involved not only in short term induction of disease resistance but also in more persistent responses. When these persistent effects are not associated with immediate induction of resistance they may be referred to as priming[174]: the resistance mechanism is set so that it can be activated more rapidly than in the unprimed state. Priming may be triggered by chemicals or pathogens and it may persist between generations[175,176] .

The target genes in priming encode PTI receptors, dormant signalling enzymes, transcription factor regulators of defense genes including WRKY factors or the antiviral AGO2 RNA silencing co-factor[177–181]. Their expression is associated with elevated levels of activating histone marks and formation of nucleosome-depleted promoter regions. The repressive marks must be removed during the activation of defense or priming and, if RdDM is involved, the pathogen-encoded suppressors of silencing could play a role.

Conclusions and perspectives

RNA silencing is clearly important in plant defense against viral and cellular pests and pathogens. Its antiviral role is adaptive in the same way that antibody and T cell systems in mammals adapt to different pathogens: the specificity in antiviral silencing varies depending on the virus[182]. In defense against cellular pathogens, however, the specificity

of the system is determined by the host-encoded RNAs that suppress virulence factors in the pathogen – it is an innate immune system.

The various silencing pathways and the pathogen-encoded suppressor proteins, considered in isolation from the other layers of defense, fit into a co-evolutionary 'arms-race' framework[10]. According to this idea the fitness of the host is increased if it can inhibit the pathogen or neutralize its virulence system. Correspondingly the pathogen's fitness depends on its capacity to counteract the host's immune system. With the connections of RNA silencing pathways to other components of the plant immune system, however, a more complex picture emerges. RNA silencing, or indeed any of the other defense systems, do not operate independently of each other they form interaction networks with components that either reinforce or counteract each other (Figure 6a-d). These networks allow selection on the host to trade off the costs and benefits of disease resistance[183–187] and for the pathogen to balance the fitness costs and benefits of virulence[128].

This network illustrates how the strategy for crop protection using dominant R genes or transgenes is much simpler than the processes in wild species in which multiple mechanisms are likely to involved with most pathogens. To escape the limitations of the conventional dominant gene approach it will be possible to learn from immune system networks develop better and more durable strategies for disease control in crops. One approach may deploy multiple strategies in parallel, with each providing partial rather than complete protection. It may also useful in addition to aim for tolerance rather than extreme resistance to pathogens in order to reduce selection for resistance[188,189].

The types of innovation to be developed could include optimised transgenes to deliver protective sRNAs against a wide range of pests and pathogens and edited endogenous sRNA loci in the plant using CRISPR-Cas so that they produce antimicrobial sRNA. Trans-kingdom sRNA targets in plant defense genes could also be modified by CRISPR-Cas so that they are not silenced in infected plants and miRNA regulators of NLRs could be deleted to enhance basal resistance. Elucidation of RNA transport pathways will also be helpful to improve the efficiency of spray RNA protection against disease[115,116]. The delivered RNA could be packed in vesicles or in association with proteins similar to those involved in natural trans-kingdom RNA silencing in the expectation that they would be taken up efficiently.

The disadvantage of this network, post arms race, approach to crop protection is that it is more complicated than the use of dominant resistance genes. It is likely, however, there will be broader spectrum efficacy and better durability that will offset the added complexity. It would mean that the grower, like the plant, will be fine tuning RNA silencing and immunity networks to trade off the costs and benefits of disease resistance.

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Glossary (terms indicated with red lettering at first use in the main text. They are listed here in the order in which they appear in the main text)

DNA virus
Most plant viruses have RNA genomes but there are two exceptional and agriculturally important groups with DNA genomes. They are geminiviruses that replicate as DNA using DNA dependent DNA polymerase and pararetroviruses including cauliflower mosaic virus for which the replication mechanism uses reverse transcriptase.

Retrotransposons
Virus-like transposable elements in plant and other genomes. Their replication involves reverse transcriptase, like pararetroviruses. Unlike pararetroviruses, however, they are integrated into the plant nuclear genome although they have extrachromosomal phases in their replication cycle and, unlike retroviruses, they do not have an envelope gene.

Replication intermediate of RNA viruses.
The replication of plant RNA viruses uses virus-encoded RNA dependent RNA polymerase and there is an dsRNA intermediate that may often have only a transient existence.

Plasmodesmata
Membrane channels in which the plasmamembrane of adjacent plants are fused. These structures span the cell wall and they are the conduit for movement between cells of metabolites, macromolecules, viruses and viroids.

Satellite RNA
Coding or non coding RNAs that are replicated by RNA polymerases of their helper viruses and transmitted between host organisms as part of the helper virus particles. They are not part of the viral genome and have distinct nucleotide sequence identity.

Autophagy
The processes by which cells degrade and recycle their components including proteolytic mechanisms that are specifically targeted.

Target mimic RNA
RNAs designed to bind miRNAs so that they are not available to find their normal target RNA. A target mimic typically has up to three mismatches in the region that base pairs with position 10 of the miRNA. As a result of these mismatches the miRNA cannot direct AGO to cleave the target mimic and the miRNA is not available to be retargeted: it is locked in a inactive form.

Mycorrhizae
Mutualistic symbioses in which fungi colonise the roots of plants. The plant provides photosynthate derived nutrients for the fungus and the fungus mobilizes and transports mineral nutrients to the infected plant.

NLR
Nod-like receptors of pathogen-derived effectors. Direct or indirect interaction of effectors with NLRs triggers ETI most likely by stimulating components of the PTI pathway.

Membrane Tubules

Paramural tubules of fungal origin associated with mutualistic and pathogenic fungal symbionts of plants. They may be associated with molecular exchanges of the two interacting organisms.

P loop motif

The phosphate-binding loop or Walker-A domain is common in NTP- binding proteins. It is rich in glycines with conserved lysine and serine or threonine.

Synonymous sites

Positions in a coding sequence that may vary at the nucleotide level without affecting the encoded protein.

Virulence

A quantitative measure of a pathogen corresponding to the degree of pathogenicity. Virulence corresponds to the extent to which the pathogen accumulates in the infected host. There is often but not always a correlation between the virulence and the severity of disease symptoms.

Figure legends

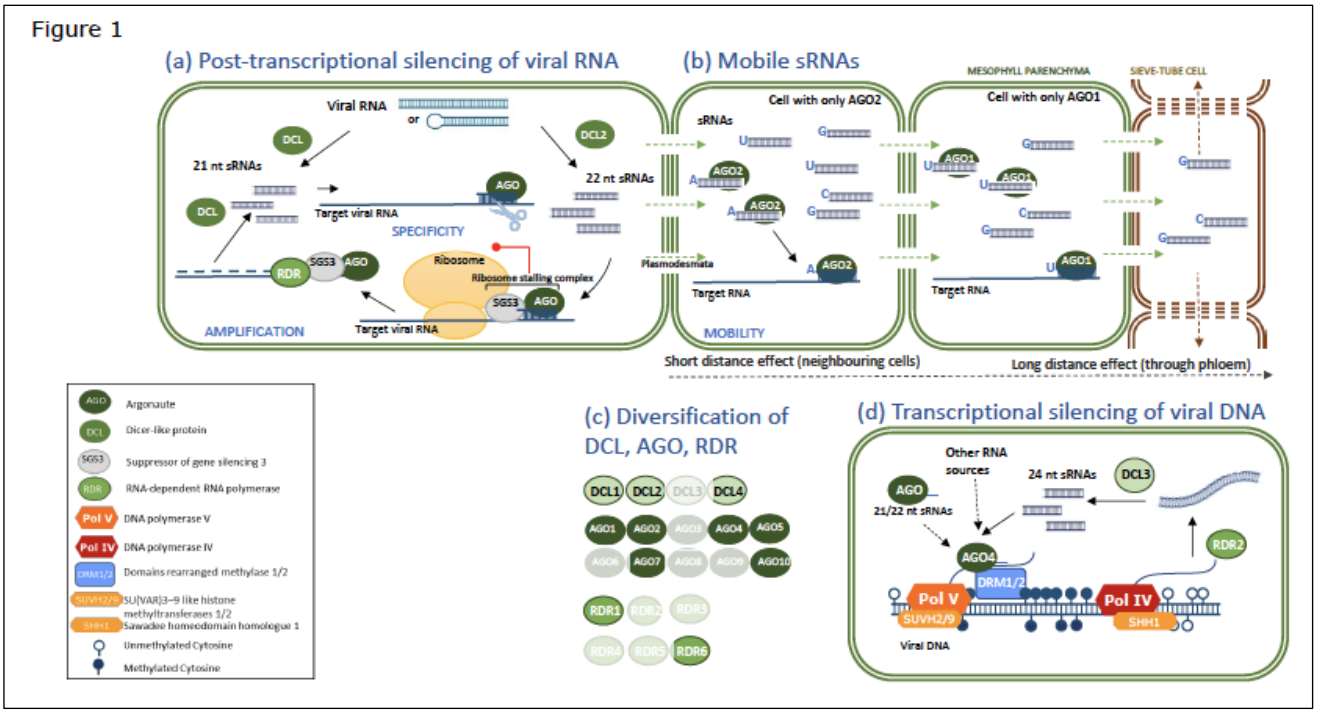

Figure 1- Variations on the canonical RNA silencing pathway in virus defense. (a) Post-transcriptional silencing of viral RNA mediated by 21/22nt sRNAs and AGO results in cleavage (scissors) or translational suppression of the targeted viral RNA. RNA silencing is **specific** because the sRNAs from viral RNA are guides for the AGO effectors of silencing to find sequence complementary targets. The DCL2-dependent 22 nt sRNAs trigger the production of SGS3-dependent secondary sRNAs, **amplifying** the silencing signal more efficiently than 21nt sRNAs. The extra nt at the 3' end protrudes from AGO and allows the recruitment of SGS3, causing ribosome stalling[17]. RDR is recruited through binding to SGS3, synthesising the duplex RNA that can be diced by DCL to produce secondary sRNAs shown here as 21nt species. **(b) sRNAs can move** cell-to-cell through plasmodesmata, to the neighbouring cells (short distance effect) or, if loaded in the phloem, reaching distal parts (long distance effect) with potential to trigger **immunity** in the recipient tissues by triggering RNA silencing (green dotted arrows depict movement)[62]. sRNAs move freely as protein-free duplexes but they are depleted according to their 5' nucleotide and the binding preference of the AGO present in that cell (5'-U and 5'-A sRNA are respective signature cargoes of AGO1 and AGO2)[59,190]. The figure illustrates the effect on the mobile sRNA population if one cell has only AGO2 and the neighbour AGO1. **(c) Diversification of proteins involved in RNA silencing**[8]. Arabidopsis has 4 DCLs, 10 AGOs and 6 RDRs. Each isoform has specialised roles in RNA silencing, and those involved in pathogen defense are highlighted. **(d) Transcriptional silencing of viral DNA** mediated by 24nt sRNAs produced from PolIV-transcript converted into dsRNA by RDR2. 24nt sRNAs loaded into AGO4 target chromatin-bound PolV-transcripts, triggering DNA methylation by recruiting DRM1/2. Feedback mechanisms to ensure transcriptional repression involve 1) methylated DNA that can be recognised by histone methylase SUVH2/9 and 2) the association of SHH1 to methylated DNA that facilitates the recruitment of PolIV[31]. Minor sRNA routes are depicted with dotted arrows and they involve various forms of dsRNAs, 21/22nt sRNAs associated with AGO1[31] or DCL-independent sRNAs produced by the action of an exonuclease[27,190].

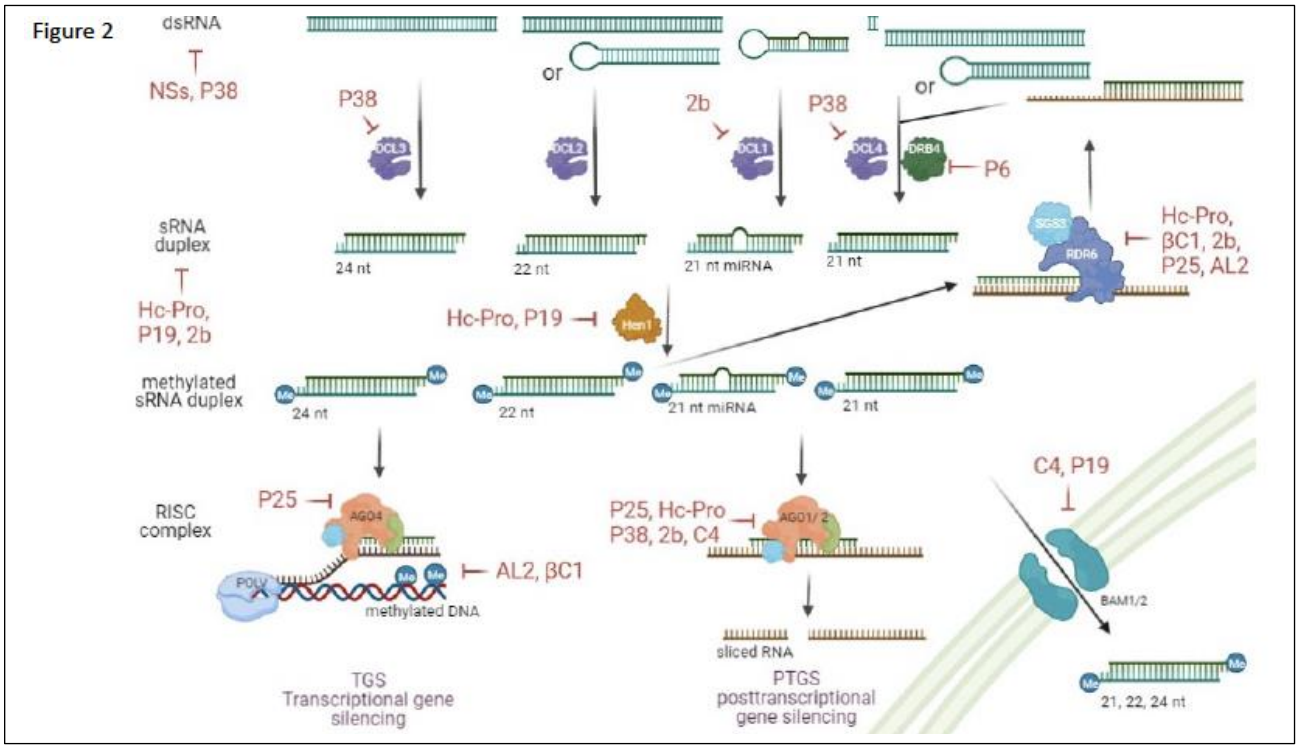

Figure 2: Mode of action of Viral suppressors of RNA silencing (VSRs). VSRs use diverse strategies to interfere with RNA silencing: binding dsRNA to prevent access to DCL, binding sRNA duplex away from AGO, mediating degradation of proteins in the RNA silencing pathway or interfiering their function, or affecting movement of an RNA silencing signal in the plasmodesmata[3].

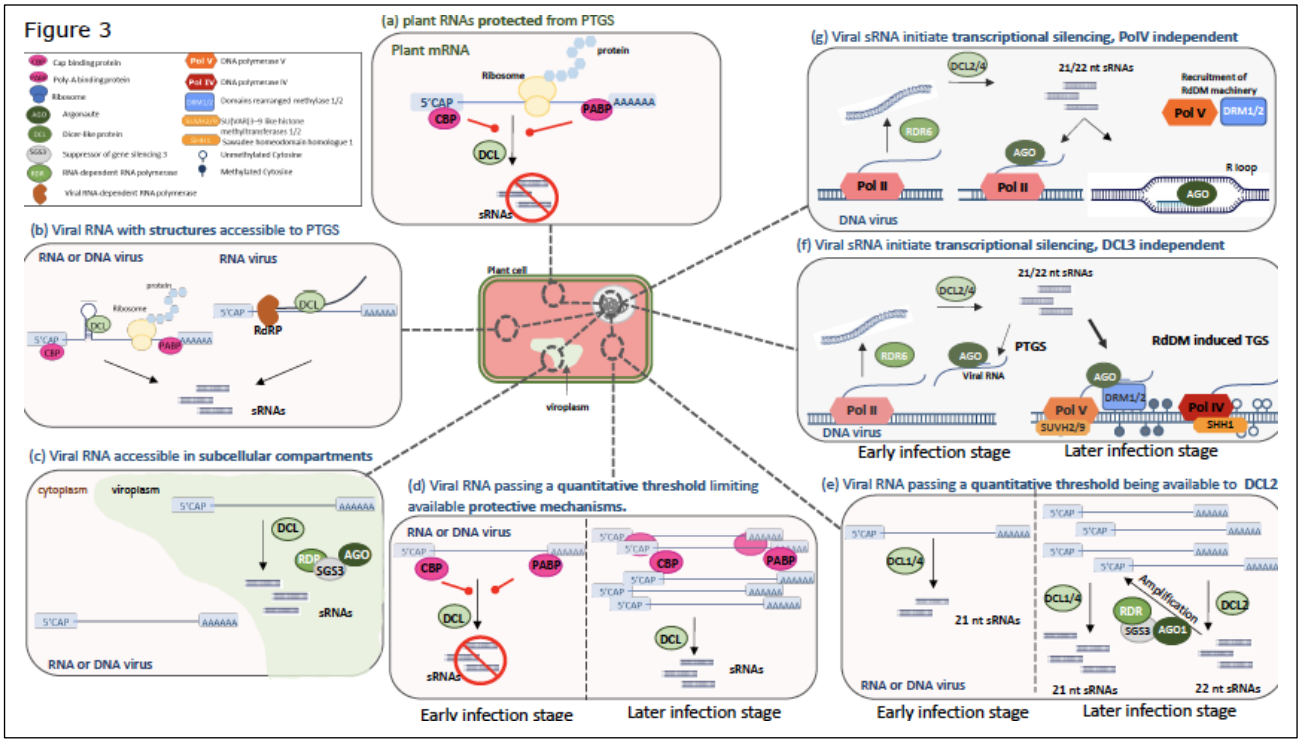

Figure 3: Strategies for *RNA silencing of viral (b-g) rather than host RNA (a)*. (a) Host plant mRNAs are accessible by ribosomes for translation but, in most instances, access by the RNA silencing machinery including RDR, SGS3 or DCL is prevented by 5'-Cap- and polyA- binding proteins associated (CBP and PABP)[44]. (b) Viral RNAs may be accessed by the RNA silencing machinery because it forms a dsRNA replication intermediate or because it has **structures**, such hairpins, that can be targeted by DCL. (c) Viral RNAs may also be available to the RNA silencing machinery because the accumulate in **subcellular compartments or viroplasm** where the CBP or PABP are not present or where there are variant forms of the RNA silencing proteins that can overcome access the viral RNA (the latter shown as example). (d) Viral RNA may increase above a certain **threshold,** so they saturate the CBP, PABP and are accessible by the RNA silencing machinery. (e) accumulation of viral RNA above this **threshold**, may provide access to DCL2, producing 22nt sRNAs that initiate the formation of **secondary sRNAs** that amplifying level of silencing. (f) 21-22 nt viral sRNAs produced by any of these routes may trigger epigenetic silencing of viral DNA independently of DCL3, through the non-canonical pathways[31]. (g) Epigenetic silencing can also be initiated independently of a PolV-transcript, by association of AGO-sRNA to a PolII transcript or by direct interaction of the sRNA with one strand of DNA[53] and recruitment of DNA methylases. The figure shows a plant cell (centre) and colour of each panel background shows the subcellular compartments (grey-nucleus, orange-cytoplasm, green-viroplasm). CBP, PABP and ribosomal translation on virus RNA shown only when relevant for the mechanism. Red arrows indicate repression.

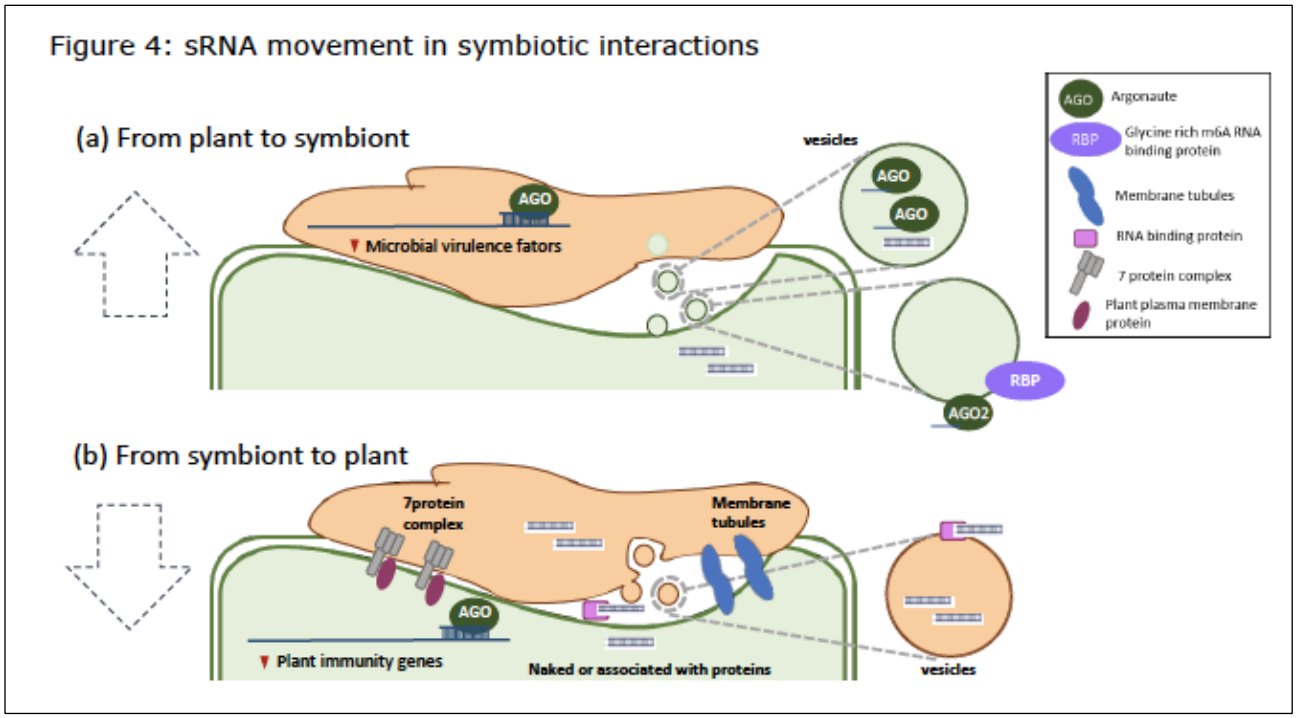

Figure 4: *sRNA movement i*n symbiotic (either pathogenic or mutualistic) interactions. (a) The trans-kingdom movement of sRNAs **from plant to symbiont** is mediated by extracellular vesicles associated with or containing AGO-sRNA nucleoproteins[109,111]. The sRNAs associated to the vesicles may target virulence factor and other transcripts in the microbe. (b) Movement of sRNA **from the symbiont to host plant** could be mediated by naked dsRNA, dsRNA associated to extracellular proteins, dsRNAs associated and/or loaded to vesicles, membrane tubules[112], or through channels formed by a seven infection-specific proteins complex that interacts with plant plasmamembrane proteins[113]. In the recipient plant cell, the microbial sRNAs associate with AGO proteins and target plant immunity genes.

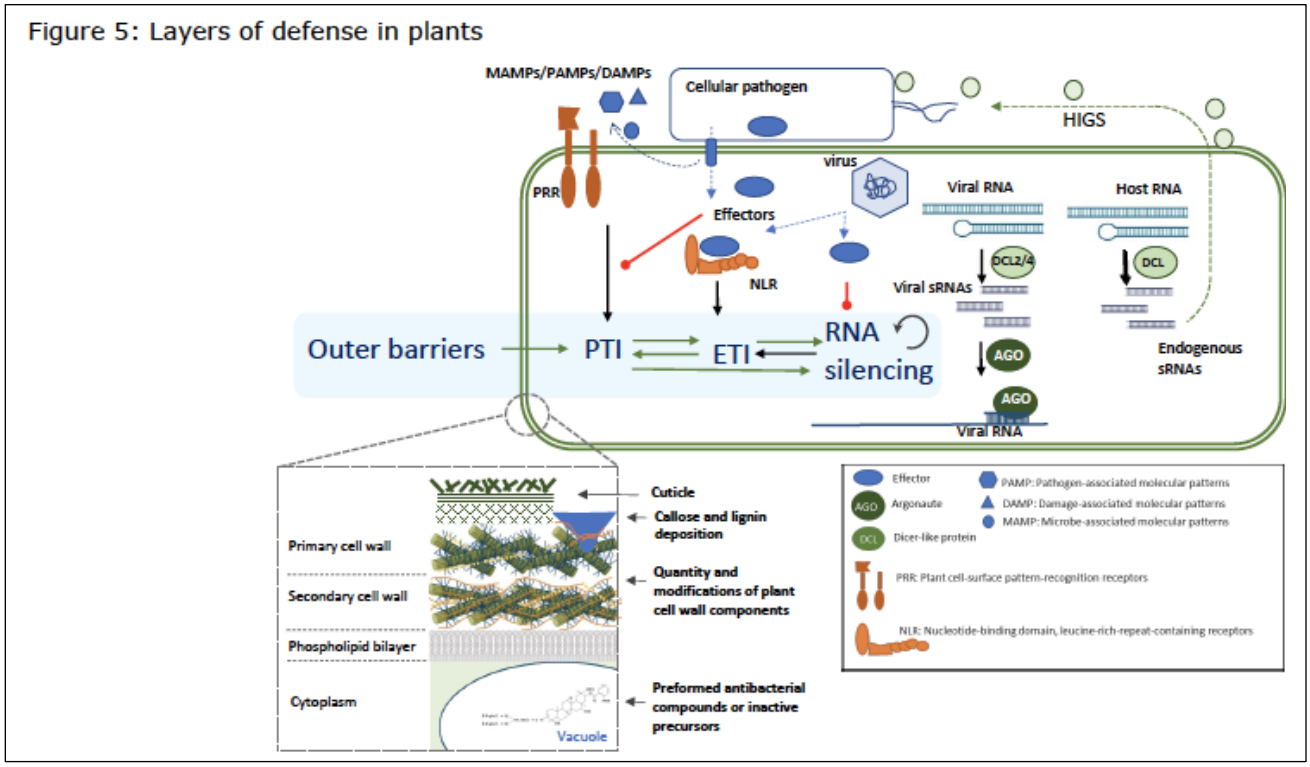

Figure 5: Layers of defense in plants. Plants have three layers of defense against viruses and other pathogens: An **outer barrier** in which physical and chemical defenses prevent infection. Some of the barriers are cuticle layer, callose and lignin deposition, change in the quantity or chemical modifications of the components of the cell wall, or storage of antimicrobe compounds or their precursors that will be release with cell damage[120,121] (zoom-out box); **inner inducible layers** in which molecules associated to pathogens (PAMPs), microbes (MAMPs) or cellular damage (DAMPs), bind to pattern recognition receptors (PRR) triggering **PTI** (PAMP-triggered immunity). The intracellular receptors (NLRs) recognise pathogenic virulence effectors, triggering **ETI** (effector-triggered immunity). In both cases the effect is cellular changes leading to disease resistance, with a reciprocal enhancement signal between ETI and PTI[125,126]. RNA silencing targets pathogenic RNA or DNA producing sRNAs that can recognise complementary viral sequences. RNA silencing interacts with the other regulatory layers, described in detail in the text and Figure 6. Red lines indicate the activity of pathogen-derived effectors to suppress ETI and PTI. Green lines indicate enhancement among the barriers, black arrows indicate an effect (enhancement or repression).

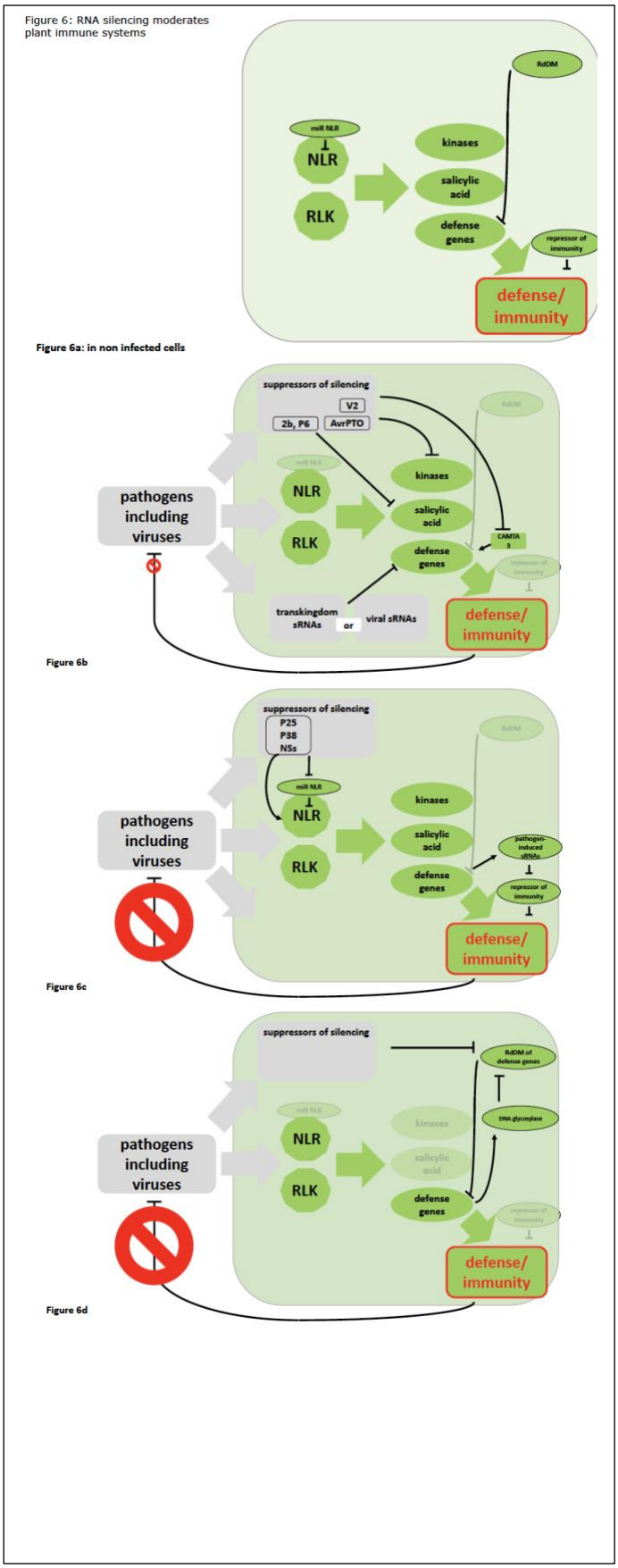

Figure 6: ***RNA silencing integrates different layers of the plant immune system.*** In non-infected plants (a) the different layers of disease resistance are partially suppressed by various repression mechanisms including miRNA-mediated or epigenetic silencing as discussed below. In infected plants low level or decreased immunity (b) (indicated by small red prohibition symbol) occurs when the trans-kingdom sRNAs block defense gene expression[101–105] or when the pathogen-encoded suppressors of silencing block the salicylic acid defense hormone (CMV (2b)[131,132], cauliflower mosaic virus (P6)[133]) or the CAMTA3 activator of defense genes (cotton leaf curl Multan geminivirus V2[134]). The AvrPTO suppressor of silencing reduces PTI by blocking a kinase[135]. Conversely there is high level increased immunity (c) in infected plants (indicated by the large red prohibition symbol) when the silencing suppressors activate ETI through NLRs (P25 of PVX[139,140], NSs of tomato spotted wilt virus[141] and P38 of TCV[142]), if they block the repressive action miR NLR[144] or if pathogen-induced sRNA targets negative regulators of immunity including auxin signalling[156], a mitochondrial protein[157], a resistance associated protein[158], a negative regulator of exocytosis[159], superoxide dismutase[160] and other mRNAs[118]. There may also be epigenetic effects (d) resulting in a high level of increased immunity in infected plants (indicated by the large red prohibition symbol) if the RdDM of defense genes [171] is blocked by suppressors of silencing or pathogen-induced expression of DNA demethylases. Defense genes affected by this process depending on the plant might include disease resistance gene *PMG1* or an immune receptor gene *RLP43*[170] or defense genes adjacent to hypomethylated transposons[170,171].

Box 1. Types of small RNAs in plants

Small RNAs can be classified as **microRNAs (miRNAs)** and **small-interfering RNAs (siRNAs)** (see table). The miRNAs[11] are produced from a non-coding protein MIR gene that is transcribed by PolII and folded back into a bulged hairpin structure that is processed by DCL1 into 21nt miRNA, or in 22nt if there is an asymmetric bulge on the miRNA strand. In plants, each MIR gene usually produces a single miRNA. Mature miRNA is loaded into the RNA interference specificity complex (RISC) (containing AGO1 or other AGOs) and, after base pairing with a target RNA, it will cleave opposite position 10-11 of the sRNA, although exceptionally it will promote mRNA degradation or arrest translation. A minor group of 22nt miRNAs are produced by DCL2, usually related to plant defence. The miRNAs are key regulators in plant growth, development, and stress response.

The siRNAs are produced from perfectly complementary dsRNA, endogenous or exogenous to the plant. These siRNAs can be further classified into different groups such as **hpRNAs, nat-siRNAs, secondary sRNAs or P4-sRNAs**.

Hairpin siRNAs (hpRNAs) are produced from long RNA folded as hairpins from inverted repeats. Some of these loci may evolve into MIR genes[9].

Natural antisense siRNAs (nat-siRNAs) are produced from overlapping transcripts originated at the same locus (cis-nat-siRNAs) or at different loci in the genome (trans-nat-siRNAs). Both hpRNA and nat-siRNA are processed by different DCLs into sRNAs of diverse sizes and regulate gene expression at posttranscriptional level[191].

Secondary siRNAs are produced following transcript targeting by a miRNA (21 or 22nt) associated with AGO1 or AGO7 (with some exceptions). The targeting process leads to dsRNA production by RDR6/SGS3. The dsRNA is cleaved by DCL4,2 or 3 producing 21, 22 or 24nt sRNAs. Not all secondary siRNAs from a transcript may have biological role regulating target genes.

Secondary sRNAs can be further classified into **phasiRNAs, tasiRNAs or easiRNAs**. The **phasiRNAs**[147] (phased secondary sRNAs) are sequentially cleaved into phased sRNAs by DCL4, starting from the end produced by the miRNA-guided cleavage. The transcripts that originate the phasiRNAs can be protein coding genes or long non coding RNAs. Some of the coding genes that originate phasiRNAs are NLRs, having phasiRNAs a role in disease resistance.

Among the phasiRNAs derived from from long non coding RNAs, there is a subgroup called **trans-acting sRNAs (tasiRNAs)**[192] because they repress other transcripts *in trans*. There are fewer than 10 known TAS loci. The long tasiRNA precursor is targeted by one or two miRNAs. The tasiRNAs produced from TAS1 and TAS2 have also been related to biotic defence or developmental regulation. Reproductive phasiRNAs were first described in monocots, being highly expressed as 21nt-phasiRNAs in premiotic anthers or as 24nt-phasiRNAs in pre-meiotic and meiotic anthers. They are important for fertility, but the molecular mechanism is not clear yet. **Epigenetically activated siRNAs (easiRNAs)**[193] are 21-22nt long, generated specifically in germ cells by DCL2 and 4, from actively transcribed transposable elements with an important role in reproduction.

Although they share similarities with phasiRNAs, it is thought that they are not closely related.

P4-siRNAs[194] (also called **heterochromatin (hc)siRNAs**) are 24nt sRNAs produced from transposable elements and other repetitive genomic regions transcribed by PolIV. These transcripts are converted in dsRNA by RDR2 and cleaved by DCL3. The sRNAs associated with AGO4 and target chromatin-bound transcripts produced by PolV. This interaction recruits DNA methyltransferases and histone methyltransferases to the DNA nearby, with the final effect of accumulation of repressive epigenetic marks and transcriptional silencing.

Box2: Viral suppressors of RNA silencing (VSRs).

Hc-Pro[4] (Helper Component Proteinase) is a multifunctional protein encoded by potato virus Y and related RNA viruses. Beyond its role blocking RNA silencing, it also affects aphid transmission of the virus, blocks the effect of the defense hormone salicylic acid and is involved in viral polyprotein maturation. In RNA silencing, HC-Pro inhibits sRNA methylation by HEN1 (HUA ENHANCER 1), sequesters dsRNA, interferes with AGO, and inhibits RDR6. It interacts with transcription factors to promote the transcription of endogenous supressors of silencing or it interacts directly with them to mis-regulate the RNA silencing pathway[4].

βC1, from the *tomato yellows leaf curl China geminivirus* (TYLCCNV), a DNA virus, promotes overexpression of an endogenous supressor of silencing calmodulin-like protein (rgsCam) and thereby prevents the production of secondary sRNAs. It also represses RDR6[41] and it interferes with TGS by limiting the amount of the S-adenosyl-methionine substrate of DNA methyltransferase[195].

AL2 from DNA genome begomovirus, also has an effect on PTGS by overexpressing rgsCam, and in TGS by affecting the methyl cycle[42,196].

C4 from a DNA virus *geminivirus*, blocks RNA silencing by interacting with the single stranded sRNA that is loaded in AGO. It also blocks the spread of the RNA silencing signal between neighbouring cellsthrough interaction with endogenous plasmodesmatal kinases (BAM1 and BAM2) that are required for cell-to-cell movement of sRNAs[34].

P19 from RNA genome tombusviruses is a very well characterised multifunctional VSR protein that has been used also as a tool to understand endogenous and viral sRNA dynamics[3]. It sequesters sRNA duplexes, inhibits HEN1-dependent methylation of sRNAs, and also interacts with BAM1 and BAM2, like C4, to prevent the spread of the silencing signal[35].

2b from RNA genome cucumoviruses is a versatile protein that targets multiple layers of RNA silencing[3]: it binds sRNAs and AGO, downregulates RDR6, AGO and DCL1 and it prevents systemic silencing. It also affects other plant defense layers such as disruption

of the SA/JA pathway, induction of the hypersensitive response or attraction of pollinators or insect vectors[137,138].

P6 from *cauliflower mosaic virus, a pararetrovirus,* is a translational transactivator protein that influences many aspects of the virus life cycle. it represses the formation of sRNAs by interacting with the nuclear protein DRB4, a cofactor required for DCL4-dependent siRNA processing. This interaction reduces but not abolish DCL4 action, allowing a balance between blocking antiviral control and host gene control through silencing. P6 also inhibits SA-induced gene expression and cell to cell movement of sRNAs[3,133].

P25 from potexvirus interacts with AGOs to promote its degradation through the proteasome pathway and also affect cell to cell movement of sRNAs by targeting RDR6/SGS3[197,198]. It also induces extensive rearrangements of actin and endomembranes and is an elicitor of hypersensitive response.

NSs from RNA tospoviruses sequesters ds-sRNA and also induces a hypersensitive response[141].

P38 from RNA carmoviruses interacts with RNA silencing at different levels: by binding to AGO1; binding and upregulating DCL1 to antagonize DCL4 and DCL 3; binding dsRNA; blocking primary siRNA biogenesis by RAV2 interaction; downregulating AGO1 via miR168 upregulation; upregulating AGO2 via blocking its silencing mediated by miR403. It also interferes with hormone signalling to promote viral infection and is an ellicitor of hypersensitive response[3].

References

1. Baulcombe, D. RNA silencing in plants. *Nature* **431**, 356–363 (2004).
2. Ding, S. W. RNA-based antiviral immunity. *Nat. Rev. Immunol.* **10**, 632–644 (2010).
3. Csorba, T., Kontra, L. & Burgyán, J. Viral silencing suppressors: Tools forged to fine-tune host-pathogen coexistence. *Virology* **479**–**480**, 85–103 (2015).
4. Valli, A. A., Gallo, A., Rodamilans, B., López-Moya, J. J. & García, J. A. The HCPro from the Potyviridae family: an enviable multitasking Helper Component that every virus would like to have. *Mol. Plant Pathol.* **19**, 744–763 (2018).
5. Gaffar, F. Y. & Koch, A. Catch me if you can! RNA silencing-based improvement of antiviral plant immunity. *Viruses* **11**, (2019).
6. Maillard, P. V, Veen, A. G. van der, Poirier, E. Z. & Sousa, C. R. e. Slicing and dicing viruses: antiviral RNA interference in mammals. *EMBO J.* **38**, e100941 (2019).
7. Bonning, B. C. & Saleh, M. C. The Interplay Between Viruses and RNAi Pathways in Insects. *Annu. Rev. Entomol.* **66**, 61–79 (2021).
8. Lee, C. H. & Carroll, B. J. Evolution and Diversification of Small RNA Pathways in Flowering Plants. *Plant Cell Physiol.* **59**, 2169–2187 (2018).
9. Borges, F. & Martienssen, R. A. The expanding world of small RNAs in plants. *Nat. Rev. Mol. Cell Biol.* **16**, 727–741 (2015).
10. Anderson, J. P. *et al.* Plants versus pathogens: an evolutionary arms race. *Funct. plant Biol.* **37**, 499 (2010).
11. Cui, J., You, C. & Chen, X. The evolution of microRNAs in plants. *Curr. Opin. Plant Biol.* **35**, 61–67 (2017).
12. Margis, R. *et al.* The evolution and diversification of Dicers in plants. *Febs Lett.* **580**, 2442–2450 (2006).
13. Wu, H. *et al.* Plant 22-nt siRNAs mediate translational repression and stress adaptation. *Nature* **581**, 89–93 (2020).
14. Cuperus, J. T. *et al.* Unique functionality of 22-nt miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in Arabidopsis. **17**, 997–1003 (2010).
15. Chen, H.-M. *et al.* 22-nucleotide RNAs trigger secondary siRNA biogenesis in plants. *Proc. Natl. Acad. Sci.* **107**, 15269–15274 (2010).
16. MacLean, D. *et al.* Evidence for large complex networks of plant short silencing RNAs. *PLoS One* **5**, e9901 (2010).
17. Iwakawa, H. oki *et al.* Ribosome stalling caused by the Argonaute-microRNA-SGS3 complex regulates the production of secondary siRNAs in plants. *Cell Rep.* **35**, 109300 (2021).
18. Wang, Z. *et al.* A novel DCL2-dependent miRNA pathway in tomato affects susceptibility to RNA viruses. *Genes Dev.* **32**, 1155–1160 (2018).
19. Wang, T. *et al.* Tomato DCL2b is required for the biosynthesis of 22-nt small RNAs, the resulting secondary siRNAs, and the host defense against ToMV. *Hortic. Res.* **5**, 62 (2018).
20. Parent, J. S., Bouteiller, N., Elmayan, T. & Vaucheret, H. Respective contributions of Arabidopsis DCL2 and DCL4 to RNA silencing. *Plant J.* **81**, 223–232 (2015).
21. Bouché, N., Lauressergues, D., Gasciolli, V. & Vaucheret, H. An antagonistic function for Arabidopsis DCL2 in development and a new function for DCL4 in

generating viral siRNAs. *EMBO J.* **25**, 3347–56 (2006).
22. Blevins, T. *et al.* Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. *Nucleic Acids Res.* **34**, 6233–6246 (2006).
23. Carbonell, A. & Carrington, J. C. Antiviral roles of plant ARGONAUTES. *Curr. Opin. Plant Biol.* **27**, 111–117 (2015).
24. Willmann, M. R. *et al.* The Functions of RNA-Dependent RNA Polymerases in Arabidopsis. in *Arabidopsis book* vol. 9 e0146 (Arabidopsis Book, 2011).
25. Coursey, T., Regedanz, E. & Bisaro, D. M. Arabidopsis RNA Polymerase V Mediates Enhanced Compaction and Silencing of Geminivirus and Transposon Chromatin during Host Recovery from Infection. *J. Virol.* **92**, 1–18 (2018).
26. Marí-Ordóñez, A. *et al.* Reconstructing de novo silencing of an active plant retrotransposon. *Nat. Genet.* **45**, 1029–39 (2013).
27. Ye, R. *et al.* A Dicer-Independent Route for Biogenesis of siRNAs that Direct DNA Methylation in Arabidopsis. *Mol. Cell* **61**, 222–235 (2016).
28. Yang, D.-L. *et al.* Dicer-independent RNA-directed DNA methylation in Arabidopsis. *Cell Res.* **26**, 66–82 (2015).
29. Huang, L. F. *et al.* An atypical RNA polymerase involved in RNA silencing shares small subunits with RNA polymerase II. *Nat. Struct. Mol. Biol.* **16**, 91–93 (2009).
30. Ream, T. S. *et al.* Subunit compositions of the RNA-silencing enzymes Pol IV and Pol V reveal their origins as specialized forms of RNA polymerase II. *Mol Cell* **33**, 192–203 (2009).
31. Matzke, M. A. & Mosher, R. A. RNA-directed DNA methylation: An epigenetic pathway of increasing complexity. *Nature Reviews Genetics* vol. 15 394–408 (2014).
32. Johnson, L. M. *et al.* SRA- and SET-domain-containing proteins link RNA polymerase V occupancy to DNA methylation. *Nature* **507**, 124–128 (2014).
33. Liu, Z. W. *et al.* The SET Domain Proteins SUVH2 and SUVH9 Are Required for Pol V Occupancy at RNA-Directed DNA Methylation Loci. *PLoS Genet.* **10**, e1003948 (2014).
34. Johnson, L. M., Law, J. A., Khattar, A., Henderson, I. R. & Jacobsen, S. E. SRA-domain proteins required for DRM2-mediated de novo DNA methylation. *PLoS Genet* **4**, e1000280 (2008).
35. Ding, S.-W. & Voinnet, O. Antiviral immunity directed by small RNAs. *Cell* **130**, 413–426 (2007).
36. Wang, L. *et al.* A virus-encoded protein suppresses methylation of the viral genome through its interaction with ago4 in the cajal body. *Elife* **9**, 1–21 (2020).
37. Raja, P., Sanville, B. C., Buchmann, R. C. & Bisaro, D. M. Viral Genome Methylation as an Epigenetic Defense against Geminiviruses. *J. Virol.* **82**, 8997–9007 (2008).
38. Rosas-Diaz, T. *et al.* A virus-targeted plant receptor-like kinase promotes cell-to-cell spread of RNAi. *Proc. Natl. Acad. Sci.* **115**, 1388–1393 (2018).
39. Garnelo Gȯmez, B. *et al.* The viral silencing suppressor P19 interacts with the receptor-like kinases BAM1 and BAM2 and suppresses the cell-to-cell movement of RNA silencing independently of its ability to bind sRNA. *New Phytol.* **229**, 1840–1843 (2021).
40. Anandalakshmi, R. *et al.* A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants. *Science* **290**, 142–144 (2000).
41. Li, F., Huang, C., Li, Z. & Zhou, X. Suppression of RNA Silencing by a Plant DNA Virus Satellite Requires a Host Calmodulin-Like Protein to Repress RDR6 Expression. *PLoS Pathog.* **10**, 11–14 (2014).

42. Yong Chung, H., Lacatus, G. & Sunter, G. Geminivirus AL2 protein induces expression of, and interacts with, a calmodulin-like gene, an endogenous regulator of gene silencing. *Virology* **460**–**461**, 108–118 (2014).
43. Shamandi, N. *et al.* Plants encode a general siRNA suppressor that is induced and suppressed by viruses. *PLoS Biol.* **13**, e1002326 (2015).
44. Liu, L. & Chen, X. RNA Quality Control as a Key to Suppressing RNA Silencing of Endogenous Genes in Plants. *Mol. Plant* **9**, 826–836 (2016).
45. Heinlein, M. Plant virus replication and movement. *Virology* **479**–**480**, 657–671 (2015).
46. Bologna, N. G. *et al.* Nucleo-cytosolic Shuttling of ARGONAUTE1 Prompts a Revised Model of the Plant MicroRNA Pathway. *Mol. Cell* **69**, 709–719 (2018).
47. Ye, R. *et al.* Cytoplasmic Assembly and Selective Nuclear Import of Arabidopsis ARGONAUTE4/siRNA Complexes. *Mol. Cell* **46**, 859–870 (2012).
48. Fang, Y. & Spector, D. L. Identification of nuclear dicing bodies containing proteins for microRNA biogenesis in living Arabidopsis plants. *Curr. Biol.* **17**, 818–823 (2007).
49. Brosseau, C. & Moffett, P. Functional and genetic analysis identify a role for Arabidopsis ARGONAUTE5 in antiviral RNA silencing. *Plant Cell* **27**, 1742–1754 (2015).
50. Zheng, X., Fahlgren, N., Abbasi, A., Berry, J. C. & Carrington, J. C. Antiviral ARGONAUTEs against turnip crinkle virus revealed by image-based trait analysis. *Plant Physiol.* **180**, 1418–1435 (2019).
51. Lindbo, J. A., Silva-Rosales, L., Proebsting, W. M. & Dougherty, W. G. Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. *Plant Cell* **5**, 1749–1759 (1993).
52. Bond, D. M. & Baulcombe, D. C. Epigenetic transitions leading to heritable, RNA-mediated de novo silencing in Arabidopsis thaliana. *Proc. Natl. Acad. Sci.* **112**, 917–922 (2015).
53. Sigman, M. J. *et al.* An siRNA-guided Argonaute protein directs RNA Polymerase V to initiate DNA methylation. *Nat. Plants* **7**, 1461–1474 (2021).
54. Mccue, A. D. *et al.* ARGONAUTE 6 bridges transposable element mRNA-derived siRNAs to the establishment of DNA methylation. *EMBO J.* **33**, 2737–2881 (2014).
55. Melnyk, C. W., Molnar, A. & Baulcombe, D. C. Intercellular and systemic movement of RNA silencing signals. *EMBO J.* **30**, 3553–3563 (2011).
56. Schwach, F., Vaistij, F. E., Jones, L. & Baulcombe, D. C. An RNA-dependent RNA-polymerase prevents meristem invasion by Potato virus X and is required for the activity but not the production of a systemic silencing signal. *Plant Physiol.* **138**, 1842–1852 (2005).
57. Havelda, Z., Hornyik, C., Crescenzi, A. & Burgyan, J. In situ characterization of Cymbidium Ringspot Tombusvirus infection-induced posttranscriptional gene silencing in Nicotiana benthamiana. *J Virol* **77**, 6082–6086 (2003).
58. Kørner, C. J. *et al.* Crosstalk between PTGS and TGS pathways in natural antiviral immunity and disease recovery. *Nat. Plants* **4**, 157–164 (2018).
59. Devers, E. A. *et al.* Movement and differential consumption of short interfering RNA duplexes underlie mobile RNA interference. *Nat. Plants* **6**, 789–799 (2020).
60. Sena, G., Jung, J. W. & Benfey, P. N. A broad competence to respond to SHORT ROOT revealed by tissue-specific ectopic expression. *Development* **131**, 2817–2826 (2004).
61. Wafula, E. *et al.* MicroRNAs from the parasitic plant Cuscuta campestris target host

messenger RNAs. *Nature* **553**, 82–85 (2018).
62. Liu, L. & Chen, X. Intercellular and systemic trafficking of RNAs in plants. *Nat. Plants* **4**, 869–878 (2018).
63. Voinnet, O., Vain, P., Angell, S. & Baulcombe, D. C. Systemic spread of sequence-specific transgene RNA degradation is initiated by localised introduction of ectopic promoterless DNA. *Cell* **95**, 177–187 (1998).
64. Vatén, A. *et al.* Callose Biosynthesis Regulates Symplastic Trafficking during Root Development. *Dev. Cell* **21**, 1144–1155 (2011).
65. Liu, J., Zhang, L. & Yan, D. Plasmodesmata-Involved Battle Against Pathogens and Potential Strategies for Strengthening Hosts. *Front. Plant Sci.* **12**, 644870 (2021).
66. Pyott, D. E. & Molnar, A. Going mobile: Non-cell-autonomous small RNAs shape the genetic landscape of plants. *Plant Biotechnol. J.* **13**, 306–318 (2015).
67. Smith, N. A., Eamens, A. L. & Wang, M. B. Viral small interfering RNAs target host genes to mediate disease symptoms in plants. *PLoS Pathog.* **7**, 1–9 (2011).
68. Shimura, H. *et al.* A viral satellite RNA induces yellow symptoms on tobacco by targeting a gene involved in chlorophyll biosynthesis using the RNA silencing machinery. *PLoS Pathog.* **7**, e1002021 (2011).
69. Adkar-Purushothama, C. R. *et al.* Small RNA derived from the virulence modulating region of the potato spindle tuber viroid silences callose synthase genes of tomato plants. *Plant Cell* **27**, 2178–2194 (2015).
70. Cao, M. *et al.* Virus infection triggers widespread silencing of host genes by a distinct class of endogenous siRNAs in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 14613–14618 (2014).
71. Pitzalis, N. *et al.* Turnip mosaic virus in oilseed rape activates networks of sRNA-mediated interactions between viral and host genomes. *Commun. Biol.* **3**, 1–16 (2020).
72. Fei, Y., Nyikó, T. & Molnar, A. Non-perfectly matching small RNAs can induce stable and heritable epigenetic modifications and can be used as molecular markers to trace the origin and fate of silencing RNAs. *Nucleic Acids Res.* **49**, 1900–1913 (2021).
73. Yang, Y. *et al.* Tomato yellow leaf curl virus intergenic siRNAs target a host long noncoding RNA to modulate disease symptoms. *PLoS Pathog.* **15**, 1–22 (2019).
74. Döring, T. F. & Chittka, L. Visual ecology of aphids—a critical review on the role of colours in host finding. *Arthropod. Plant. Interact.* **1**, 3–16 (2007).
75. Timmons, L. & Fire, A. Specific interference by ingested dsRNA. *Nature* **395**, 854 (1998).
76. Banerjee, S. *et al.* RNA interference: A novel source of resistance to combat plant parasitic nematodes. *Front. Plant Sci.* **8**, 1–8 (2017).
77. Hudzik, C., Hou, Y., Ma, W. & Axtell, M. J. Exchange of small regulatory rnas between plants and their pests. *Plant Physiol.* **182**, 51–62 (2020).
78. Nowara, D. *et al.* HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen Blumeria graminis. *Plant Cell* **22**, 3130–3141 (2010).
79. Pliego, C. *et al.* Host-induced gene silencing in barley powdery mildew reveals a class of ribonuclease-like effectors. *Mol. Plant-Microbe Interact.* **26**, 633–642 (2013).
80. Zhang, H. *et al.* Functional characterization of calcineurin homologs PsCNA1/PsCNB1 in Puccinia striiformis f. sp. tritici using a host-induced RNAi system. *PLoS One* **7**, 1–8 (2012).
81. Koch, A. *et al.* Host-induced gene silencing of cytochrome P450 lanosterol C14α-

demethylase-encoding genes confers strong resistance to Fusarium species. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 19324–19329 (2013).
82. Panwar, V., McCallum, B. & Bakkeren, G. Host-induced gene silencing of wheat leaf rust fungus Puccinia triticina pathogenicity genes mediated by the Barley stripe mosaic virus. *Plant Mol. Biol.* **81**, 595–608 (2013).
83. Ghag, S. B., Shekhawat, U. K. S. & Ganapathi, T. R. Host-induced post-transcriptional hairpin RNA-mediated gene silencing of vital fungal genes confers efficient resistance against Fusarium wilt in banana. *Plant Biotechnol. J.* **12**, 541–553 (2014).
84. Andrade, C. M., Tinoco, M. L. P., Rieth, A. F., Maia, F. C. O. & Aragão, F. J. L. Host-induced gene silencing in the necrotrophic fungal pathogen Sclerotinia sclerotiorum. *Plant Pathol.* **65**, 626–632 (2016).
85. Wang, M. *et al.* Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nat. Plants* **2**, 16151 (2016).
86. Chen, W. *et al.* Host-induced silencing of Fusarium culmorum genes protects wheat from infection. *J. Exp. Bot.* **67**, 4979–4991 (2016).
87. Govindarajulu, M., Epstein, L., Wroblewski, T. & Michelmore, R. W. Host-induced gene silencing inhibits the biotrophic pathogen causing downy mildew of lettuce. *Plant Biotechnol. J.* **13**, 875–883 (2015).
88. Jahan, S. N. *et al.* Plant-mediated gene silencing restricts growth of the potato late blight pathogen Phytophthora infestans. *J. Exp. Bot.* **25**, 265–288 (2015).
89. Hou, Y. *et al.* A Phytophthora Effector Suppresses Trans-Kingdom RNAi to Promote Disease Susceptibility. *Cell Host Microbe* **25**, 153–165 (2019).
90. Helber, N. *et al.* 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 (2011).
91. Singla-Rastogi, M. *et al.* Plant Small RNA Species Direct Gene Silencing in Pathogenic Bacteria as well as Disease Protection. *Bioarxiv* 5.
92. He, B. *et al.* RNA-binding proteins contribute to small RNA loading in plant extracellular vesicles. *Nat. Plants* **7**, 342–352 (2021).
93. Cai, Q. *et al.* Plants send small RNAs in extracellular vesicles to silence virulence genes. *Science* **360**, 1126–1129 (2018).
94. Zhang, T. *et al.* Cotton plants export microRNAs to inhibit virulence gene expression in a fungal pathogen. *Nat. Plants* **2**, 1–6 (2016).
95. Navarro, L., Jay, F., Nomura, K., He, S. Y. & Voinnet, O. Suppression of the microRNA pathway by bacterial effector proteins. *Science* **321**, 964–967 (2008).
96. Vetukuri, R. R., Whisson, S. C. & Grenville-Briggs, L. J. Phytophthora infestans effector Pi14054 is a novel candidate suppressor of host silencing mechanisms. *Eur. J. Plant Pathol.* **149**, 771–777 (2017).
97. Qiao, Y. *et al.* Oomycete pathogens encode RNA silencing suppressors. *Nat. Genet.* **45**, 1–6 (2013).
98. Qiao, Y., Shi, J., Zhai, Y., Hou, Y. & Ma, W. Phytophthora effector targets a novel component of small RNA pathway in plants to promote infection. *Proc. Natl. Acad. Sci.* **15**, 5850–5855 (2015).
99. Xiong, Q. *et al.* Phytophthora Suppressor of RNA Silencing 2 Is a Conserved RxLR Effector that Promotes Infection in Soybean and Arabidopsis thaliana. *Mol. Plant-Microbe Interact.* **27**, 1379–1389 (2014).
100. Yin, C. *et al.* A novel fungal effector from Puccinia graminis suppressing RNA silencing and plant defense responses. *New Phytol.* **222**, 1561–1572 (2019).

101. Weiberg, A. *et al.* Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. *Science* **342**, 118–123 (2013).
102. Wang, B. *et al.* Puccinia striiformis f. sp. tritici microRNA-like RNA 1 (Pst-milR1), an important pathogenicity factor of Pst, impairs wheat resistance to Pst by suppressing the wheat pathogenesis-related 2 gene. *New Phytol.* **215**, 338–350 (2017).
103. Jian, J. & Liang, X. One small RNA of Fusarium graminearum targets and silences CEBiP gene in common wheat. *Microorganisms* **7**, 1–12 (2019).
104. Dunker, F. *et al.* Oomycete small RNAs bind to the plant RNA-induced silencing complex for virulence. *Elife* **9**, 1–23 (2020).
105. Hu, X., Persson Hodén, K., Liao, Z., Åsman, A. & Dixelius, C. Phytophthora infestans Ago1-associated miRNA promotes potato late blight disease. *New Phytol.* **233**, 443–457 (2022).
106. Baldrich, P. *et al.* Plant extracellular vesicles contain diverse small RNA species and are enriched in 10- to 17-nucleotide "Tiny" RNAs. *Plant Cell* **31**, 315–324 (2019).
107. Ren, B., Wang, X., Duan, J. & Ma, J. Rhizobial tRNA-derived small RNAs are signal molecules regulating plant nodulation. *Science* **365**, 919–922 (2019).
108. Wong-Bajracharya, J. *et al.* The ectomycorrhizal fungus Pisolithus microcarpus encodes a microRNA involved in cross-kingdom gene silencing during symbiosis. *Proc. Natl. Acad. Sci.* **119**, e2103527119 (2022).
109. Cai, Q. *et al.* Message in a Bubble: Shuttling Small RNAs and Proteins between Cells and Interacting Organisms Using Extracellular Vesicles. *Annu. Rev. Plant Biol.* **72**, 497–524 (2021).
110. Rutter, B. D. & Innes, R. W. Growing pains: addressing the pitfalls of plant extracellular vesicle research. *New Phytol.* **228**, 1505–1510 (2020).
111. Karimi, H. Z. *et al.* Arabidopsis Apoplastic Fluid Contains sRNA- and Circular RNA-Protein Complexes that Are Located Outside Extracellular Vesicles. *Plant Cell* **in press**, (2022).
112. Roth, R. *et al.* Arbuscular cell invasion coincides with extracellular vesicles and membrane tubules. *Nat. Plants* **5**, 204–211 (2019).
113. Ludwig, N. *et al.* A cell surface-exposed protein complex with an essential virulence function in Ustilago maydis. *Nat. Microbiol.* **6**, 722–730 (2021).
114. Laurie, J. D., Linning, R. & Bakkeren, G. Hallmarks of RNA silencing are found in the smut fungus Ustilago hordei but not in its close relative Ustilago maydis. *Curr. Genet.* **53**, 49–58 (2008).
115. Taliansky, M. *et al.* RNA-based technologies for engineering plant virus resistance. *Plants* **10**, 1–19 (2021).
116. Qiao, L. *et al.* Spray-induced gene silencing for disease control is dependent on the efficiency of pathogen RNA uptake. *Plant Biotechnol. J.* **19**, 1756–1768 (2021).
117. Johnson, N. R., de Pamphilis, C. W. & Axtell, M. J. Compensatory sequence variation between trans-species small rnas and their target sites. *Elife* **8**, 1–17 (2019).
118. Rose, L. E., Overdijk, E. J. R. & van Damme, M. Small RNA molecules and their role in plant disease. *Eur. J. Plant Pathol.* **153**, 115–128 (2019).
119. Serrano, M., Coluccia, F., Torres, M., L'Haridon, F. & Métraux, J. P. The cuticle and plant defense to pathogens. *Front. Plant Sci.* **5**, 1–8 (2014).
120. Bacete, L., Mélida, H., Miedes, E. & Molina, A. Plant cell wall-mediated immunity: cell wall changes trigger disease resistance responses. *Plant J.* **93**, 614–636

(2018).
121. Osbourn, A. E. Preformed Antimicrobial Compounds and Plant Defense against Fungal Attack. *Plant Cell* **8**, 1821–1831 (1996).
122. Kourelis, J. & Van Der Hoorn, R. A. L. Defended to the nines: 25 years of resistance gene cloning identifies nine mechanisms for R protein function. *Plant Cell* **30**, 285–299 (2018).
123. Bentham, A. R. *et al.* A molecular roadmap to the plant immune system. *J. Biol. Chem.* **295**, 14916–14935 (2020).
124. Yuan, M. *et al.* Pattern-recognition receptors are required for NLR-mediated plant immunity. *Nature* **592**, 105–109 (2021).
125. Ngou, B. P. M., Ahn, H. K., Ding, P. & Jones, J. D. G. Mutual potentiation of plant immunity by cell-surface and intracellular receptors. *Nature* **592**, 110–115 (2021).
126. Tian, H. *et al.* Activation of TIR signalling boosts pattern-triggered immunity. *Nature* **598**, 500–503 (2021).
127. Hou, S., Liu, Z., Shen, H. & Wu, D. Damage-associated molecular pattern-triggered immunity in plants. *Front. Plant Sci.* **10**, 646 (2019).
128. Sacristán, S. & García-Arenal, F. The evolution of virulence and pathogenicity in plant pathogen populations. *Mol. Plant Pathol.* **9**, 369–384 (2008).
129. Agorio, A. & Vera, P. ARGONAUTE4 is required for resistance to Pseudomonas syringae in Arabidopsis. *Plant Cell* **19**, 3778–3790 (2007).
130. López, A., Ramírez, V., García-Andrade, J., Flors, V. & Vera, P. The RNA silencing enzyme RNA polymerase V is required for plant immunity. *PLoS Genet.* **7**, 1002434 (2011).
131. Zhou, T. *et al.* Domains of the cucumber mosaic virus 2b silencing suppressor protein affecting inhibition of salicylic acid-induced resistance and priming of salicylic acid accumulation during infection. *J. Gen. Virol.* **95**, 1408–1413 (2014).
132. Ji, L. H. & Ding, S. W. The suppressor of transgene RNA silencing encoded by Cucumber mosaic virus interferes with salicylic acid-mediated virus resistance. *Mol. Plant-Microbe Interact.* **14**, 715–724 (2001).
133. Love, A. J. *et al.* Cauliflower mosaic virus Protein P6 Inhibits Signaling Responses to Salicylic Acid and Regulates Innate Immunity. *PLoS One* **7**, e47535 (2012).
134. Wang, Y. *et al.* A calmodulin-binding transcription factor links calcium signaling to antiviral RNAi defense in plants. *Cell Host Microbe* **29**, 1393–1406 (2021).
135. Shan, L. *et al.* Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. *Cell Host Microbe* **4**, 17–27 (2008).
136. He, J. *et al.* Structural analysis of Phytophthora suppressor of RNA silencing 2 (PSR2) reveals a conserved modular fold contributing to virulence. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 8054–8059 (2019).
137. Wu, D. *et al.* Viral effector protein manipulates host hormone signaling to attract insect vectors. *Cell Res.* **27**, 402–415 (2017).
138. Ziebell, H. *et al.* Cucumber mosaic virus and its 2b RNA silencing suppressor modify plant-aphid interactions in tobacco. *Sci. Rep.* **1**, 1–7 (2011).
139. Malcuit, I. *et al.* The 25-kDa movement protein of PVX elicits Nb-mediated hypersensitive cell death in potato. *Mol. Plant-Microbe Interact.* **12**, 536–543 (1999).
140. Voinnet, O., Lederer, C. & Baulcombe, D. C. A viral movement protein prevents spread of the gene silencing signal in Nicotiana benthamiana. *Cell* **103**, 157–167 (2000).

141. de Ronde, D. *et al.* Analysis of Tomato spotted wilt virus NSs protein indicates the importance of the N-terminal domain for avirulence and RNA silencing suppression. *Mol. Plant Pathol.* **15**, 185–195 (2014).
142. Ren, T., Qu, F. & Morris, T. J. HRT Gene Function Requires Interaction between a NAC Protein and Viral Capsid Protein to Confer Resistance to Turnip Crinkle Virus. *Plant Cell* **12**, 1917–1926 (2000).
143. Zhai, J. *et al.* MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, trans-acting siRNAs. *Genes Dev* **25**, 2540–2553 (2011).
144. Shivaprasad, P. V *et al.* A microRNA superfamily regulates nucleotide binding site-leucine-rich repeats and other mRNAs. *Plant Cell* **24**, 859–874 (2012).
145. Liu, J. *et al.* The miR9863 Family Regulates Distinct Mla Alleles in Barley to Attenuate NLR Receptor-Triggered Disease Resistance and Cell-Death Signaling. *PLoS Genet.* **10**, e1004755 (2014).
146. Li, F. *et al.* MicroRNA regulation of plant innate immune receptors. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 1790–1795 (2011).
147. Liu, Y., Teng, C., Xia, R. & Meyers, B. C. PhasiRNAs in Plants: Their Biogenesis, genic sources, and roles in stress responses, development, and reproduction. *Plant Cell* **32**, 3059–3080 (2020).
148. Canto-Pastor, A. *et al.* Enhanced resistance to bacterial and oomycete pathogens by short tandem target mimic RNAs in tomato. *Proc Natl Acad Sci U S A* **116**, 2755–2760 (2019).
149. Deng, Y. *et al.* A role for small RNA in regulating innate immunity during plant growth. *PLoS Pathog.* **14**, e1006756 (2018).
150. Cui, C. *et al.* A Brassica miRNA Regulates Plant Growth and Immunity through Distinct Modes of Action. *Mol. Plant* **13**, 231–245 (2020).
151. Vaucheret, H., Mallory, A. C. & Bartel, D. P. AGO1 homeostasis entails coexpression of MIR168 and AGO1 and preferential stabilization of miR168 by AGO1. *Mol. Cell* **22**, 129–136 (2006).
152. Xie, Z., Kasschau, K. D. & Carrington, J. C. Negative Feedback Regulation of Dicer-Like1 in Arabidopsis by microRNA-Guided mRNA Degradation. *Curr. Biol.* **13**, 784–789 (2003).
153. Harvey, J. J. W. *et al.* An antiviral defense role of AGO2 in plants. *PLoS One* **6**, e14639 (2011).
154. Jiang, N., Meng, J., Cui, J., Sun, G. & Luan, Y. Function identification of miR482b, a negative regulator during tomato resistance to Phytophthora infestans. *Hortic. Res.* **5**, 9 (2018).
155. Nakahara, K. S. *et al.* Tobacco calmodulin-like protein provides secondary defense by binding to and directing degradation of virus RNA silencing suppressors. *Proc. Natl. Acad. Sci.* **109**, 10113–10118 (2012).
156. Navarro, L. *et al.* A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* **312**, 436 (2006).
157. Katiyar-Agarwal, S. *et al.* A pathogen-inducible endogenous siRNA in plant immunity. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 18002–18007 (2006).
158. Campo, S. *et al.* Identification of a novel microRNA (miRNA) from rice that targets an alternatively spliced transcript of the Nramp6 (Natural resistance-associated macrophage protein 6) gene involved in pathogen resistance. *New Phytol.* **199**, 212–227 (2013).
159. Zhang, X. *et al.* Arabidopsis Argonaute 2 Regulates Innate Immunity via

miRNA393*-Mediated Silencing of a Golgi-Localized SNARE Gene, MEMB12. *Mol. Cell* **42**, 356–366 (2011).
160. Li, Y. *et al.* Identification of microRNAs involved in pathogen-associated molecular pattern-triggered plant innate immunity. *Plant Physiol.* **152**, 2222–2231 (2010).
161. Yang, Z. *et al.* Jasmonate Signaling Enhances RNA Silencing and Antiviral Defense in Rice. *Cell Host Microbe* **28**, 89–103 (2020).
162. Wu, J. *et al.* Viral-inducible Argonaute18 confers broad-spectrum virus resistance in rice by sequestering a host microRNA. *Elife* **2015**, 1–19 (2015).
163. Dowen, R. H. *et al.* Widespread dynamic DNA methylation in response to biotic stress. *Proc. Natl. Acad. Sci. U. S. A.* **109**, E2183–E2191 (2012).
164. Yu, A. *et al.* Dynamics and biological relevance of DNA demethylation in Arabidopsis antibacterial defense. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 2389–2394 (2013).
165. Akimoto, K. *et al.* Epigenetic inheritance in rice plants. *Ann. Bot.* **100**, 205–217 (2007).
166. Corrêa, R. L. *et al.* Viral Fitness Determines the Magnitude of Transcriptomic and Epigenomic Reprograming of Defense Responses in Plants. *Mol. Biol. Evol.* **37**, 1866–1881 (2020).
167. Jaskiewicz, M., Conrath, U. & Peterhälnsel, C. Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Rep.* **12**, 50–55 (2011).
168. Schillheim, B. *et al.* Sulforaphane modifies histone H3, unpacks chromatin, and primes defense. *Plant Physiol.* **176**, 2395–2405 (2018).
169. Jin, H. *et al.* Salicylic acid-induced transcriptional reprogramming by the HAC-NPR1-TGA histone acetyltransferase complex in Arabidopsis. *Nucleic Acids Res.* **46**, 11712–11725 (2018).
170. Halter, T. *et al.* The arabidopsis active demethylase ros1 cis-regulates defense genes by erasing DNA methylation at promoter-regulatory regions. *Elife* **10**, e62994 (2021).
171. López Sánchez, A., Stassen, J. H. M., Furci, L., Smith, L. M. & Ton, J. The role of DNA (de)methylation in immune responsiveness of Arabidopsis. *Plant J.* **88**, 361–374 (2016).
172. Geng, S. *et al.* DNA methylation dynamics during the interaction of wheat progenitor Aegilops tauschii with the obligate biotrophic fungus Blumeria graminis f. sp. tritici. *New Phytol.* **221**, 1023–1035 (2019).
173. Satgé, C. *et al.* Reprogramming of DNA methylation is critical for nodule development in Medicago truncatula. *Nat. Plants* **2**, 1–10 (2016).
174. Conrath, U., Beckers, G. J. M., Langenbach, C. J. G. & Jaskiewicz, M. R. Priming for Enhanced Defense. *Annu. Rev. Phytopathol.* **53**, 97–119 (2015).
175. Luna, E., Bruce, T. J. A., Roberts, M. R., Flors, V. & Ton, J. Next-generation systemic acquired resistance. *Plant Physiol.* **158**, 844–853 (2012).
176. Slaughter, A. *et al.* Descendants of primed Arabidopsis plants exhibit resistance to biotic stress. *Plant Physiol.* **158**, 835–843 (2012).
177. Ando, S. *et al.* Priming for enhanced ARGONAUTE2 activation accompanies induced resistance to cucumber mosaic virus in Arabidopsis thaliana. *Mol. Plant Pathol.* **22**, 19–30 (2021).
178. Beckers, G. J. M. *et al.* Mitogen-Activated protein kinases 3 and 6 are required for full priming of stress responses in Arabidopsis thaliana. *Plant Cell* **21**, 944–953 (2009).

179. Pick, T., Jaskiewicz, M., Peterhänsel, C. & Conrath, U. Heat shock factor HsfB1 primes gene transcription and systemic acquired resistance in Arabidopsis. *Plant Physiol.* **159**, 52–55 (2012).
180. Baum, S. *et al.* Isolation of open chromatin identifies regulators of systemic acquired Resistance. *Plant Physiol.* **181**, 817–833 (2019).
181. Furci, L. *et al.* Identification and characterisation of hypomethylated DNA loci controlling quantitative resistance in Arabidopsis. *Elife* **8**, e40655 (2019).
182. Voinnet, O. RNA silencing as a plant immune system against viruses. *Trends Genet.* **17**, 449–459 (2001).
183. Tamborski, J. & Krasileva, K. V. Evolution of Plant NLRs: From Natural History to Precise Modifications. *Annu. Rev. Plant Biol.* **71**, 355–378 (2020).
184. Zhao, M., Meyers, B. C., Cai, C., Xu, W. & Ma, J. Evolutionary Patterns and Coevolutionary Consequences of MIRNA Genes and MicroRNA Targets Triggered by Multiple Mechanisms of Genomic Duplications in Soybean. *Plant Cell* **27**, 546–562 (2015).
185. González, V. M. V. M., Müller, S., Baulcombe, D. C. & Puigdomènech, P. Evolution of NBS-LRR Gene Copies among Dicot Plants and its Regulation by Members of the miR482/2118 Superfamily of miRNAs. *Mol. Plant* **8**, 329–331 (2015).
186. Deng, Y. *et al.* Epigenetic regulation of antagonistic receptors confers rice blast resistance with yield balance. *Science* **355**, 962–965 (2017).
187. Karasov, T. L., Chae, E., Herman, J. J. & Bergelson, J. Mechanisms to mitigate the trade-off between growth and defense. *Plant Cell* **29**, 666–680 (2017).
188. Kover, P. X. & Schaal, B. A. Genetic variation for disease resistance and tolerance among Arabidopsis thaliana accessions. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 11270–11274 (2002).
189. Poland, J. A., Balint-Kurti, P. J., Wisser, R. J., Pratt, R. C. & Nelson, R. J. Shades of gray: the world of quantitative disease resistance. *Trends Plant Sci.* **14**, 21–29 (2009).
190. Mi, S. J. *et al.* Sorting of small RNAs into Arabidopsis argonaute complexes is directed by the 5 ′ terminal nucleotide. *Cell* **133**, 116–127 (2008).
191. Borsani, O., Zhu, J. K., Verslues, P. E., Sunkar, R. & Zhu, J. K. Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. *Cell* **123**, 1279–1291 (2005).
192. Allen, E., Xie, Z., Gustafson, A. M. & Carrington, J. C. microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell* **121**, 207–221 (2005).
193. Borges, F. *et al.* Transposon-derived small RNAs triggered by miR845 mediate genome dosage response in Arabidopsis. *Nat. Genet.* **50**, 186–192 (2018).
194. Bond, D. M. & Baulcombe, D. C. Small RNAs and heritable epigenetic variation in plants. *Trends Cell Biol.* **24**, 100–107 (2014).
195. Yang, X. *et al.* Suppression of Methylation-Mediated Transcriptional Gene Silencing by βC1-SAHH Protein Interaction during Geminivirus-Betasatellite Infection. *PLOS Pathog.* **7**, e1002329 (2011).
196. Hao, L., Wang, H., Sunter, G. & Bisaro, D. M. Geminivirus AL2 and L2 Proteins Interact with and Inactivate SNF1 Kinase. *Plant Cell* **15**, 1034–1048 (2003).
197. Bayne, E. H., Rakitina, D. V., Morozov, S. Y. & Baulcombe, D. C. Cell-to-cell movement of Potato Potexvirus X is dependent on suppression of RNA silencing. *Plant J.* **44**, 471–482 (2005).
198. Chiu, M. H., Chen, I. H., Baulcombe, D. C. & Tsai, C. H. The silencing suppressor P25 of Potato virus X interacts with Argonaute1 and mediates its degradation

through the proteasome pathway. *Mol. Plant Pathol.* **11**, 641–649 (2010).

Highlighted references

13. Wu, H. *et al.* Plant 22-nt siRNAs mediate translational repression and stress adaptation. *Nature* **581**, 89–93 (2020).

22nt sRNAs trigger amplification of silencing and in this report there is evidence also for an effect on translational regulation.

36. Wang, L. *et al.* A virus-encoded protein suppresses methylation of the viral genome through its interaction with ago4 in the cajal body. *eLife* **9**, 1–21 (2020).

Previous evidence implicated RdDM in virus defense and the Cajal body in RdDM. This paper links the two through an interaction of a VSR (V2 of TYLCV) with AGO4 in the Cajal Body.

53. Sigman, M. J. *et al.* An siRNA-guided Argonaute protein directs RNA Polymerase V to initiate DNA methylation. *Nat. Plants* **7**, 1461–1474 (2021).

The initial establishment phase is the least well understood stage in RdDM. This paper provides important evidence that an AGO protein is binds to the DNA target and that it recruits PolV.

59. Devers, E. A. *et al.* Movement and differential consumption of short interfering RNA duplexes underlie mobile RNA interference. *Nature Plants* **6**, 789–799 (2020).

Clear evidence that mobile silencing is mediated by AGO free sRNA duplexes that are bound by AGO proteins in the recipient cell.

111. Karimi, H. Z. *et al.* Arabidopsis Apoplastic Fluid Contains sRNA- and Circular RNA-Protein Complexes that Are Located Outside Extracellular Vesicles. *Plant Cell* **in press**, (2022).

Controversial findings leading to the conclusion that trans-kingdom RNA may be transported outside of extracellular vesicles. This extracellular RNA is enriched for m^6A and associated with GLYCINE-RICH RNA-BINDING PROTEIN 7 (GRP7) and small RNA-binding 42 protein ARGONAUTE2 (AGO2).

134. Wang, Y. *et al.* A calmodulin-binding transcription factor links calcium signaling to antiviral RNAi defense in plants. *Cell Host Microbe* **29**, 1393–1406 (2021).

A clear illustration of how VSRs mediate connections between RNA silencing and other defense systems.

136. He, J. *et al.* Structural analysis of Phytophthora suppressor of RNA silencing 2 (PSR2) reveals a conserved modular fold contributing to virulence. *Proceedings of*

the National Academy of Sciences of the United States of America **116**, 8054–8059 (2019).

This paper provides structural information about a suppressor of silencing from the late blight Phytophthora oomycete.

148. Canto-Pastor, A. *et al.* Enhanced resistance to bacterial and oomycete pathogens by short tandem target mimic RNAs in tomato. *Proc Natl Acad Sci U S A* **116**, 2755–2760 (2019).

154. Jiang, N., Meng, J., Cui, J., Sun, G. & Luan, Y. Function identification of miR482b, a negative regulator during tomato resistance to Phytophthora infestans. *Horticulture Research* **5**, 9 (2018).

This paper and 145 illustrate how miRNA mediated targeting of NLRs leads to suppression of basal resistance in non infected plants and, therefore, how there is the potential for pathogen encoded suppressors of silencing to enhance immunity in infected plants.

162. Wu, J. *et al.* Viral-inducible Argonaute18 confers broad-spectrum virus resistance in rice by sequestering a host microRNA. *eLife* **2015**, 1–19 (2015).

Another nice illustration of how there is a complex network of defense systems in plants. RNA silencing is an important effector mechanism and integrator of the different systems.

170. Halter, T. *et al.* The arabidopsis active demethylase ros1 cis-regulates defense genes by erasing DNA methylation at promoter-regulatory regions. *Elife* **10**, 1–62 (2021).

New findings showing the importance of active DNA demethylation in PTI and basal resistance.

181. Furci, L. *et al.* Identification and characterisation of hypomethylated DNA loci controlling quantitative resistance in Arabidopsis. *Elife* **8**, e40655 (2019).

Not directly related to RNA silencing but illustrating a connection and priming of immunity by prior exposure to a pathogen.

Group		Alternative names	subgroup	Size (nt)	precursor	DCL	AGO	target	actio n	Ref
miRNA				21, (22,24)	MIR gene	DCL1, (DCL2,3)	AGO1 (AGO2,7,10)	Cytoplasmic RNA	PTGS	[11]
siRNAs	hp-siRNAs	IR-sRNAs, endo-sRNAs		21,22,24	Hairpin from IR	DCL2,3,4	AGO1,4	RNA	PTGS (and TGS)	[9]
	nat-siRNAs		Cis-nat-siRNAs, trans-nat-siRNAs	21,22,24	Overlapping transcripts	DCL2,3,4	AGO1,4	RNA	PTGS (and TGS)	[191]
	Secondary sRNAs		phasiRNAs ,tasiRNAs, easiRNAs	21, 22, 24	dsRNA by SGS3/RDR6 from a transcript targeted by a miRNA	DCL2,4	AGO1	Cytoplasmic RNA	PTGS	[147,193]
	P4- sRNAs	het-sRNAs, rasiRNAs, 24nt-sRNAs		24	PolIV transcript, converted in dsRNA by RDR2	DCL3	AGO4 (AGO6,9)	Nuclear PolV RNA	TGS	[31]