MECHANISMS OF PLANT RESISTANCE TO VIRUSES

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Abstract | Plants have evolved in an environment rich with microorganisms that are eager to capitalize on the plants' biosynthetic and energy-producing capabilities. There are approximately 450 species of plant-pathogenic viruses, which cause a range of diseases. However, plants have not been passive in the face of these assaults, but have developed elaborate and effective defence mechanisms to prevent, or limit, damage owing to viral infection. Plant resistance genes confer resistance to various pathogens, including viruses. The defence response that is initiated after detection of a specific virus is stereotypical, and the cellular and physiological features associated with it have been well characterized. Recently, RNA silencing has gained prominence as an important cellular pathway for defence against foreign nucleic acids, including viruses. These pathways function in concert to result in effective protection against virus infection in plants.

INNATE IMMUNITY
The suite of host responses to pathogens that result in rapid defence without requiring prior stimulation.

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Plants and viruses enter into various relationships that do not necessarily result in damage to the host (BOX 1). If a pathogenic virus succeeds in infecting a plant, a selection of INNATE IMMUNITY mechanisms might defeat the virus. Should the virus circumvent these defence mechanisms, disease outbreaks and epidemics occur. For example, during the 1990s, cassava production in Uganda was devastated by cassava mosaic geminiviruses, resulting in famine-related deaths[1]. It is estimated that on the African continent in 2003 more than 19 million tons of cassava, valued at more than US$1.9 billion, was lost[1]. Clearly, understanding plant defence is required to develop approaches to protect the world's food supply.

Perhaps the best-characterized mechanism of plant antiviral defence is mediated by resistance *(R)* genes. *R* genes confer resistance to organisms including viruses, bacteria, fungi and even nematodes[2]. R proteins and their signal-transduction molecules are strikingly similar to the components of the animal innate immune system. How these conserved signalling modules function in plants is the subject of intense research.

Of increasing interest in plant antiviral strategies is the role of RNA silencing, an ancient cellular mechanism of defence against foreign nucleic acids that also functions in gene regulation. The RNA-silencing pathway is found in organisms that are separated by millions of years of evolution. How do R proteins and RNA silencing interact to limit viral pathogenesis? We review recent advances in the field of plant defence against viruses.

R genes

Plant *R* genes confer resistance to many pathogens, including viruses. Here, we describe the responses mediated by these genes and how R proteins probably function.

***R*-gene mediated responses.** Each *R* gene confers resistance to a specific pathogen (BOX 1). The first phenotype of defence in most *R*-gene-mediated resistance responses is the hypersensitive response (HR). The HR includes programmed cell death (PCD), which occurs in cells at the site of infection and manifests as discrete necrotic lesions in otherwise phenotypically normal tissue (FIG. 1a). The virus is usually confined to the lesion and to the cells immediately surrounding it and fails to spread from lesions into adjacent

Box 1 | **Virulence and avirulence**

The interactions between plants and microorganisms are complex, and several relationships have been described. A given microorganism can only infect selected plant species. On the one hand, if a microorganism cannot infect a plant species, the plant species is described as a non-host. Failure to infect a non-host species is usually due to basal defences, which include physical barriers to infection such as the cell wall, waxy cuticle and bark, as well as the production of several antimicrobial compounds. If, on the other hand, a microorganism can infect and replicate in a plant species, the plant is referred to as a host for that microorganism. For interactions in which the pathogen infects and replicates in the host to produce disease, the pathogen is described as virulent, the plant is described as susceptible and the interaction is termed compatible.

Plants have resistance (*R*) genes that confer resistance to specific pathogens. For example, the *Arabidopsis thaliana RCY1* gene confers resistance to the Y strain of cucumber mosaic virus, but not to the O strain. When the Y strain of CMV infects *RCY1*-containing plants, a defence response is initiated, which restricts the virus to the infection site and prevents disease. The virus is an avirulent pathogen on these resistant plants and this is termed an incompatible interaction. The pathogen molecule that specifically elicits R-protein-mediated responses is the avirulence (Avr) determinant. Avr proteins are usually necessary for successful infection and are almost invariably virulence factors in a susceptible host.

healthy tissues. The second phenotype of *R*-gene-mediated resistance — systemic acquired resistance (SAR) (FIG. 1c) — occurs in tissues that are distant from the initial infection site and renders them immune to infection by the same or closely related pathogens. Interestingly, SAR is durable and can last for several weeks. SAR is characterized by the increased expression of several genes, named pathogenesis-related genes, that encode antimicrobial compounds.

R-protein structure. In the last decade, several *R* genes that confer resistance to unrelated plant viruses have been cloned (TABLE 1). Strikingly, they all belong to the NB-ARC-LRR superfamily of plant *R* genes. The nucleotide-binding site contains three motifs that are required for nucleotide binding in other ATP/GTP-binding proteins. The nucleotide-binding (NB) site domain and adjacent sequences of *R* genes are similar to the equivalent regions of the metazoan cell-death genes *Apaf-1* and *CED4* (REF. 3) and are therefore referred to as the NB-ARC domains. ATPase activity has been shown for two R proteins but the role of ATP hydrolysis in R-protein function is unclear[4]. Leucine-rich repeats (LRRs) are imperfect repeats that are involved in protein–protein interactions and protein–ligand interactions. The LRRs of mammalian Toll-like receptors (TLRs) interact with pathogen-derived molecules to initiate defence responses[5]. In addition, plant R proteins of this superfamily bear striking resemblance to mammalian NODS (nucleotide-binding oligomerization domain), which are intracellular NB-ARC-LRR proteins involved in defence[6].

The NB-ARC-LRR R proteins can be further subdivided based on the structure of their N termini. TIR-NB-ARC-LRR proteins have an N-terminal Toll-interleukin-1 receptor (TIR) homology domain[7,8] (TABLE 1). The TIR domain is conserved in plant R proteins and receptors that function in metazoan innate immunity, such as *Drosophila melanogaster* Toll receptor and the mammalian TLRs and interleukin-1 receptor[9]. The other subfamily of cloned viral *R* genes encodes CC-NB-ARC-LRR proteins that have an N-terminal coiled-coil (CC) domain (TABLE 1). TIR, NB-ARC and LRR domains are also found in molecules that are important for animal innate immunity, indicating that innate defence mechanisms evolved in a common ancestor preceding the divergence of plant and animal lineages.

Although these R proteins are similar, they confer resistance to highly divergent viruses. For example, *Arabidopsis thaliana RCY1* (resistance to C strain Y1) and *HRT* (HR to turnip crinkle virus) are allelic and encode proteins that share 91% similarity[10] but confer resistance to unrelated viruses: cucumber mosaic virus (CMV, a cucumovirus) and turnip crinkle virus (TCV, a carmovirus), respectively. Any model developed to describe R-protein recognition of a pathogen must take into account the striking molecular similarity but functional divergence of R proteins.

R-protein-domain function. Extensive structure–function analyses of R-protein domains have been carried out. Mutations in all three domains of the *Nicotiana glutinosa* N protein compromise resistance to tobacco mosaic virus (TMV), indicating that each domain might have important roles in pathogen recognition and/or signalling[11]. Similar results have been obtained from studies of *Solanum tuberosum* Rx1 R-protein-mediated resistance to potato virus X (PVX)[12].

There have also been attempts to identify the domains that confer recognition specificity on R proteins. Domain swaps between different alleles of the flax *L* gene, which confers resistance to a fungal pathogen, indicated that both the LRR and the TIR domains have roles in the recognition of R proteins[13]. We have also found evidence supporting this from our analysis of *RCY1* and several of its alleles, including *HRT* and *RPP8* (an *R* gene that confers resistance to the oomycete *Peronospora parasitica*). Several residues in these proteins might be undergoing positive selection, and most of them are found within the LRR domain (J.L.M.S. and S.P.D.-K., unpublished).

An interesting feature of *R*-gene regulation is the alternative splicing of transcripts of TIR-NB-ARC-LRR members[14]. The N_S transcript of the *N* gene encodes the full-length N protein and the alternatively spliced N_L transcript encodes a putative protein that lacks almost the entire LRR domain. In uninfected plants, there is an excess of N_S, but in the presence of TMV, N_L levels increase dramatically within seven hours of infection[15]. However, this is a transient change, and basal transcript levels are rapidly restored. Modulation of *N* mRNA splicing is necessary to confer complete resistance to TMV. Alternative splicing has also been proposed to occur for *S. tuberosum R* gene *Y-1* (REF. 8). In addition, alternative splicing occurs in the *L6* and *RPS4* resistance genes that are involved in defence against non-viral pathogens[14].

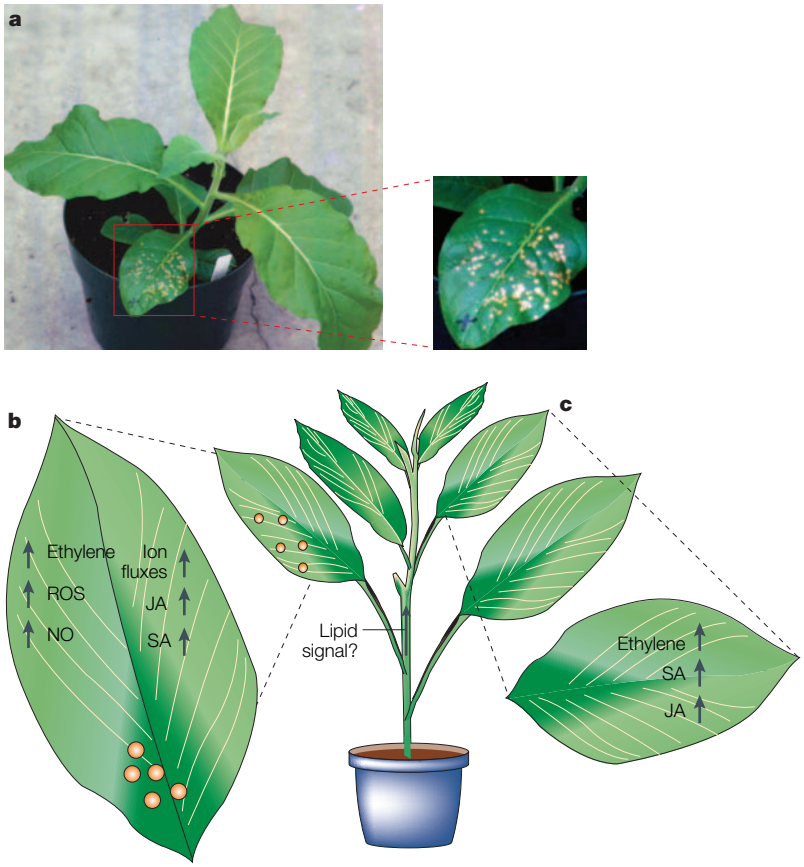


Figure 1 | **Local and systemic resistance mediated by resistance (*R*) genes. a** | Picture of an *N*-containing plant showing typical hypersensitive response (HR) necrotic lesions upon tobacco mosaic virus infection. The uninfected upper leaves are symptom-free and do not contain virus. **b** | During resistance, several signalling molecules are locally induced. **c** | Subsequent to the HR, systemic acquired resistance is induced in distal uninfected tissue. The systemic signal is currently unknown, but is thought to be lipid-derived. JA, jasmonic acid; NO, nitric oxide; SA, salicylic acid; ROS, reactive oxygen species.

The plant proteins that are encoded by splice variants have not been detected. However, several functions have been proposed for the proteins that might be encoded by these alternative transcripts. First, the predicted truncated protein encoded by N_L, a TIR-NB-ARC-only protein, might interact with full-length N protein after infection. This interaction could enable signal transduction leading to resistance. Second, interaction between the N_L and N_S proteins could disrupt protein–protein interactions that are required to inhibit ectopic R-protein signalling. Either explanation could account for the loss of resistance observed in plants that have incorrectly spliced *N* transcripts[15].

The involvement of splice variants in regulating TIR protein function is not limited to plants, as mammalian TLRs also require alternative splicing for function. TLR alternative splicing results in a loss of the TIR domain, as opposed to the loss of the LRR domain in plants. Interestingly, truncated mammalian TLR4 inhibits lipopolysaccharide-mediated signals, and its transcript is induced by its cognate pathogen, in common with the plant system[14,16].

Intramolecular rearrangements within R proteins have also been shown to be involved in recognition[12,17]. Co-expression of CC-NB-ARC and LRR domains or CC and NB-ARC-LRR domains of *S. tuberosum* Rx1 results in a HR in the presence of the PVX coat-protein Avr (avirulence) determinant[17] (BOX 1), mimicking the function of the intact Rx1 protein. The CC-NB-ARC and LRR or the CC and NB-ARC-LRR co-immunoprecipitate, but this interaction is abrogated in the presence of the coat protein[17]. These results indicate that there are specific interactions between the domains of *S. tuberosum* Rx1 and that the coat protein disturbs the native conformation of Rx1. It is probable that molecular interactions within *S. tuberosum* Rx1 hold the protein in a conformation that is poised for signalling but is inhibited from doing so. Addition of the Avr determinant releases the inhibition and allows defence signalling[17]. Evidence for intramolecular interactions has also been obtained for the tomato Mi-1 R protein, which confers resistance to a nematode[18].

Recognition of Avr determinants. Any protein component of a virus can function as the specific Avr determinant to elicit resistance mediated by a given *R* gene (TABLE 1). Despite the availability of several cloned *R* genes and their cognate Avr determinants (TABLE 1), progress in understanding how pathogen Avr proteins are recognized has been slow. Initially, receptor–ligand models were proposed to describe the interactions between R and Avr proteins (FIG. 2a). However, this simple model does not apply to any viral R–Avr pair examined to date, although it has been shown to apply in two cases of bacterial and one case of fungal resistance[2]. A more sophisticated model of R–Avr interactions invokes the involvement of R-protein-containing complexes. The 'guard hypothesis', originally proposed by Van der Biezen and Jones[19], postulates that R proteins (guards) are constitutively associated with host cellular proteins (guardees) that are required by pathogens for infection (FIG. 2b). On infection, the pathogen causes modifications to the guardee that are detected by the guard (FIG. 2c). Any protein modification that can alter the quaternary structure of the guardee could result in detection of the pathogen. This activates the guard to initiate a signalling cascade that culminates in the resistance response[19].

To date, the most convincing evidence for the guard hypothesis has been found in *A. thaliana* bacterial R-protein–Avr systems. In an elegant series of experiments, RIN4 (RPM1-interacting protein 4) was identified as a cellular protein that is required for the resistance to *Pseudomonas syringae* pv. *tomato* that is mediated by RPM1 and RPS2. *P. syringae* pv. *tomato* carries several Avr proteins[20–22]. RIN4 (guardee) is modified in various ways, depending on the Avr that it associates with, and these modifications then serve to activate the corresponding R protein (guard). A second example is the cleavage of the *A. thaliana* kinase PBS1 (guardee) by the cysteine protease AvrPphB from *P. syringae* pv. *tomato*, which results in activation of *RPS5* (guard)-mediated resistance[23].

Table 1 | **Cloned plant resistance (*R*) genes and the viral proteins that their proteins recognize**

Gene	Plant	R protein Structure	Virus	Avr determinant	Ref.
N	*Nicotiana* sp.	TIR-NB-ARC-LRR	Tobacco mosaic virus	Replicase	7
Rx1	*Solanum tuberosum*	CC-NB-ARC-LRR	Potato virus X	Coat protein	60
Rx2	*S. tuberosum*	CC-NB-ARC-LRR	Potato virus X	Coat protein	103
HRT	*Arabidopsis thaliana* ecotype Dijon-17	CC-NB-ARC-LRR	Turnip crinkle virus	Coat protein	104
RCY1	*A. thaliana* ecotype C24	CC-NB-ARC-LRR	Cucumber mosaic virus strain Y	Coat protein	10
Sw-5	*Lycopersicon* sp.	CC-NB-ARC-LRR	Tomato spotted wilt virus	RNA-dependent RNA polymerase	105
Y-1	*S. tuberosum*	TIR-NB-ARC-LRR	Potato virus Y	?	8
Tm-2²	*Lycopersicon* sp.	CC-NB-ARC-LRR	Tomato mosaic virus	Movement protein	61

CC, coiled coil; LRR, leucine-rich repeat; NB, nucleotide-binding; TIR, Toll-interleukin-1 receptor.

The viral R-protein–Avr system that lends the greatest support to the guard hypothesis is the HRT–TCV pair. The TCV coat protein is the Avr determinant for *HRT*-mediated resistance responses (TABLE 1), and it interacts with a host transcription factor, TCV-interacting protein (TIP)[24]. This interaction is required for *HRT*-elicited defence responses[24]. Although a direct interaction between HRT and TIP has not been reported, TCV coat protein inhibits the nuclear localization of TIP[25]. It is possible that HRT detects the altered cellular distribution of TIP. TIP might therefore be the guardee of the guard protein HRT.

The guard hypothesis is appealing because it explains how hosts can overcome pathogen evolution, as resistance mediated by guard-protein function does not rely on direct interactions between the R and Avr proteins. The same R protein could recognize the presence of multiple Avr proteins through either a single or multiple guardees. This is true for RPM1, which is activated by both AvrB and AvrRpm through their interactions with RIN4 (REF. 21). This paradigm might also hold true for other R proteins, and could explain the unexpectedly small number of *R* genes in the sequenced *A. thaliana* genome[26]. There are approximately 200 *R*-gene-like sequences in the 125 Mb *A. thaliana* genome that confer resistance to thousands of pathogens[26]. Furthermore, this model accounts for the functional divergence of the structurally similar R proteins. Despite its attractiveness, one caveat of the guard hypothesis is that the virulence function of the guardees identified has not been proven to date.

R-protein complexes. Recent data supports the existence of R-protein-containing complexes. The conserved heat shock protein 90 (Hsp90), a molecular CHAPERONE, is required for the resistance that is mediated by the R proteins Rx1 and N (TABLE 1), as well as RPM1 and RPS2 (REFS 27–30). There is a close association of Hsp90 with R proteins, possibly within a larger complex[28,30]. Hsp90 in turn associates with SGT1 and Rar1 (REFS 28–30), two proteins that are required for the function of several *R* genes (see below). The function of the Hsp90–R-protein complex is not clear. Hsp90 could be involved in the conformational regulation of these complexes with the help of the co-chaperones SGT1 and Rar1. Hsp90 might also be required for the stability of R proteins, perhaps preventing their degradation, as some R proteins, including Rx1, fail to accumulate in the absence of Hsp90 (REFS 27,28). Another possibility is that Hsp90 and other chaperones regulate the conformation of R proteins within complexes, perhaps facilitating intramolecular rearrangements.

Signalling in R-gene-mediated resistance. The early host responses following pathogen detection include changes in ion fluxes, activation of signalling pathways (especially kinase cascades), gross alteration of transcriptional profiles, generation of reactive oxygen species (ROS) and production of nitric oxide (NO). These immediate changes are subsequently followed by altered cellular activities with the recruitment of several hormones that then participate in defence. The typical outcome of these responses is PCD of infected cells. This suite of defence responses is termed the HR (FIG. 1).

There is a transient Ca^{2+} signature change upon infection with avirulent pathogens that is required for effective defence[31]. Changes in ion fluxes are believed to activate several kinase cascades, for example, Ca^{2+} binding by calcium-dependent protein kinases triggers phosphorylation relays[31]. These cascades are important for signal transduction during defence. Two putative kinase cascades that are required for N-mediated defence have also recently been described[32,33]. The wounding-induced protein kinase (WIPK) and salicylic-acid-induced protein kinase (SIPK) are mitogen-activated protein kinases that are upregulated in defence[34]. When activated, WIPK and SIPK induce expression of defence-related genes, including several transcription factors that are members of the WRKY family of plant-specific transcription factors (contain one or more WRKY domains, which are characterized by the heptapeptide WRKYGQK) and MYB transcription factors[35].

Not surprisingly, microarray analyses have revealed complex and massive changes in transcriptional activity in cells and tissues undergoing the HR. For example,

CHAPERONES
Chaperones are a large group of highly conserved proteins that assist other polypeptides in folding, stabilize large complexes and ensure correct localization of proteins. Although constitutively expressed, they are typically induced to higher levels by stress and are crucial for cell survival under these conditions.

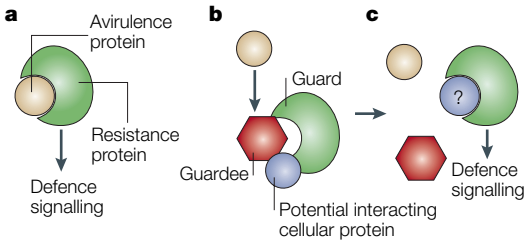


Figure 2 | **The receptor–ligand hypothesis versus the guard hypothesis. a** | The receptor–ligand hypothesis predicts that resistance proteins detect pathogen infection by directly interacting with avirulence proteins, triggering defence signalling. **b** | In the guard hypothesis, resistance proteins 'guard' cellular proteins, 'guardees'. These guardees are the targets of avirulence proteins and are posited to be required for successful infection by the pathogen. The guard and the guardee dynamically interact and there might be other protein(s) in the complex. **c** | Upon modification of the guardee by the avirulence protein, the interaction between the guard and guardee is altered and the guard triggers a signalling cascade that leads to defence.

in *A. thaliana* infected with CMV, 444 genes show altered rates of transcription[36]. It will perhaps be more informative to determine if the actual protein profile of these tissues reflect the modulation of transcripts.

ROS generated during the HR also induce expression of defence-related genes, in addition to initiating the PCD that is associated with the HR, and probably increase crosslinking of the cell wall[37]. Many of the details regarding production of ROS during defence have been determined[38]. The enzyme respiratory burst oxidase homologue, which is homologous to the mammalian NADPH oxidase *gp91^{phox}*, is important for the generation of ROS during defence[38,39]. The mitochondrial alternative oxidase (AOX) functions to limit ROS production[40] and has also been implicated in hormone-induced resistance[41]. However, the role of AOX in defence has not yet been clarified[41,42].

In conjunction with ROS, the small molecule NO is required for pathogen-induced PCD in the HR[43]. NO also induces the expression of defence-related genes[44], although the function of some of these gene products in resistance to viruses is unclear. Although NO is produced constitutively as a by-product of cellular metabolism[45], it is also induced upon pathogen infection[43,44]. There are several possible sources of NO during defence. *A. thaliana NITRIC OXIDE SYNTHASE 1 (AtNOS1)* is a homologue of the snail NOS and catalyses the abscisic acid (a plant hormone)-induced production of NO[46]. Mutants that map to *atnos1* show reduced resistance to bacterial pathogens[47]. The cooperation between ROS and NO in plant defence is similar to the mammalian system, where phagocytic cells use ROS and NO species in defence against microbial pathogens[48].

Hormones are also important effectors of defence against pathogens, and their roles are well documented. Jasmonic acid[49], ethylene[50] and salicylic acid[51] seem to be the most important hormones involved in defence. Extensive crosstalk occurs between these pathways[52]. This is not surprising if one considers that multiple pathways that reinforce each other ensure a successful outcome of the defence response. Crosstalk between pathways also fine-tunes the regulation of defence responses and enables feedback to occur.

Usually, the first visible outcome of R-protein–Avr interaction is the HR PCD (FIG. 1). The HR PCD might induce subsequent defence responses[53], but it must be tightly regulated. Mutants that show uncontrolled spread of lesions have been identified[54]. If PCD is misregulated, uncontrolled death that becomes pathologic to the host could be expected. The signal that initiates the spread of PCD in these mutants and the signal that is required to attenuate the spreading of PCD in the normal HR have not yet been identified. Recent evidence indicates that AUTOPHAGY might be the mechanism by which PCD is limited to infection sites[55]. This suggests that there is a signal that moves out of the primary HR site into surrounding tissue and causes adjacent cells to die, and that autophagy is required to remove this signal in the cells that surround the HR site.

Apoptosis, a type of PCD in animals, is regulated by a class of cysteine proteases called the caspases. Although many of the physiological characteristics of plant PCD closely resemble apoptosis[53], comprehensive genome searches have failed to identify plant proteins with significant similarity to caspases. However, several early studies found that caspase inhibitors and animal apoptosis proteins could affect plant PCD[56]. Consequently, there has been a large effort to identify the protease mediators of plant apoptosis-like PCD. A protease that cleaves its targets in a caspase-like manner was recently found to be active early in the N–TMV interaction[57]. The enzymatic activity of this protease is required for HR-associated PCD. Another protease, vacuolar processing enzyme, has a caspase-like activity that is required for PCD during *N*-induced defence to TMV[58] and to other virulent and avirulent pathogens[59]. These results are interesting because they indicate that PCD is required for resistance in the N–TMV system, in contrast to other systems such as Rx1–PVX and Tm-2^2–ToMV (tomato mosaic virus) (REFS 60,61) (TABLE 1).

In addition to proteases, ubiquitin-mediated protein degradation is implicated in disease resistance. There are many excellent recent reviews on proteasome-mediated protein degradation in plants[62]. In brief, ubiquitin is attached to the substrate for degradation through a series of ubiquitin-conjugating enzymes, E1 to E3. Ubiquitin-tagged proteins are then degraded by the 26S proteasome. One class of E3 is a complex made up of Skp1/Cullin/F-box (SCF) proteins. Several proteins that are associated with the ubiquitination machinery have been shown to be required for some resistance pathways, including Skp1 and SGT1 as well as the SGT1-associated protein Rar1 (REF. 63). The COP9 signalosome (CSN) is a protein complex that has many diverse cellular functions associated with

AUTOPHAGY
Autophagy, meaning to eat (phagy) oneself (auto), is the cellular pathway for the degradation of both long-lived proteins and organelles that is involved in cellular development, innate immunity and starvation responses. Substrates are packaged in double-membraned vesicles and targeted to lysosomes and vacuoles for processing and degradation.

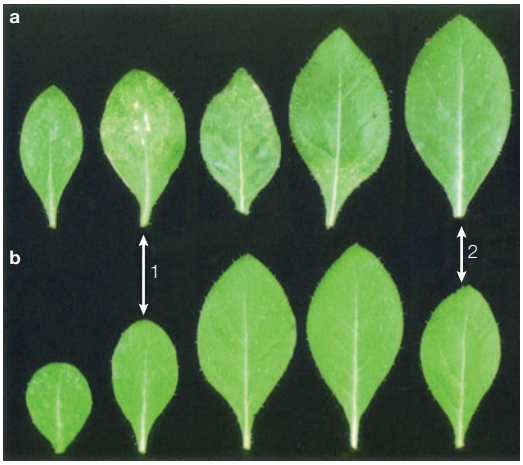


Figure 3 | **Recovery.** Row **a** shows *Nicotiana clevelandii* plants infected with tomato black ring nepovirus. The plants recover from a primary infection (1) and are resistant to a secondary infection (2). The leaves in row **b** are mock-infected. Leaves are arranged from left to right in decreasing age. Reprinted with permission from REF. 102 © (1997) American Association for the Advancement of Science.

protein degradation and modification[64]. The CSN interacts with SCF complexes[64] and also with SGT1 (REFS 65,66), and is required for N-mediated resistance[66]. In addition, the F-box protein COI1, which is essential for jasmonic-acid signalling, is part of the SCF complex, SCF^{COI1} (REF. 67).

Although it is apparent that protein degradation is an important component of resistance, the substrates of all these degradative molecules remain to be elucidated. This is an important question, as these targets must be negative regulators of defence and PCD. Their identification would shed light on the actual mechanisms by which defence responses occur. These negative regulators could also be modified by genetic engineering to produce plants with greater resistance under field conditions.

Following the HR, a secondary defence response, SAR, is activated. Salicylic acid is produced during the HR and then appears later in uninfected tissues that are developing SAR, however, salicylic acid is not the systemic signal for SAR[68]. Recent evidence indicates that the systemic signal might be lipid-derived (FIG. 1). A tobacco salicylic-acid-binding protein, SABP2, has high affinity for salicylic acid and shows lipase activity upon binding it[69]. An *A. thaliana* mutant defective in *induced resistance 1-1* (*dir1-1*) lacks the systemic signal that induces SAR[70], but salicylic-acid levels are unaffected. *DIR1* encodes a putative apoplastic lipid transfer-like protein (LTP)[70]. The *A. thaliana* mutants *enhanced disease susceptibility 1* (*eds1*) and *phytoalexin-deficient 4* (*pad4*) have defects in putative lipases[71]. However, unlike *dir1-1*, these mutants fail to accumulate salicylic acid upon infection and are therefore believed to act upstream of salicylic acid in both local defence and SAR.

DICER
DICER is a member of the RNase III family of nucleases that specifically cleave dsRNAs. DICER processes long dsRNA into siRNAs of 21–23 nucleotides.

RNA silencing

For decades, scientists and farmers have observed that diseased, virus-infected plants grow new, symptom-free leaves. In fact, the same (or a related) virus cannot infect the healthy upper leaves of these plants. This is described as 'recovery', and RNA silencing has emerged as a potential mechanism of recovery[72] (FIG. 3). RNA silencing is well characterized and conserved among plants, fungi, insects and animals[73] (BOX 2).

Initiation of RNA silencing of viruses. Double-stranded RNA (dsRNA) is the trigger for RNA silencing[73] (BOX 2). The RNA sequence that is homologous to the dsRNA trigger is degraded and the gene that encodes that RNA is effectively silenced. Both plant and animal viruses have minimized the accessibility of their replicative intermediates to host defences, so where does the dsRNA that initiates RNA silencing of viruses come from?

Most plant viruses — representing 59 of the 80 plant-virus genera — are RNA viruses[74]. These viruses encode RNA-dependent RNA polymerases (RdRPs) that, in the first steps of replication, produce opposite-sense copies of the viral genome[74]. It has been suggested that this generates many long dsRNA species that trigger RNA silencing[75,76]. However, replicative intermediates of negative-strand RNA viruses, like the human influenza virus, are coated with nucleocapsid protein[77]. Perhaps this prevents replicative intermediates from forming dsRNA[78]. An alternative hypothesis is that viral-RNA secondary structures might be the trigger for RNA silencing[72]. A third hypothesis is that viral replication generates RNA molecules that ectopically activate, and are replicated by, host RdRPs to produce dsRNA. These foreign RNA sequences could trigger viral RNA silencing[78]. It will be interesting to identify the RNA species that induce viral RNA silencing, as they might be useful not only for the engineering of virus resistant plants, but also as a tool for molecular analysis.

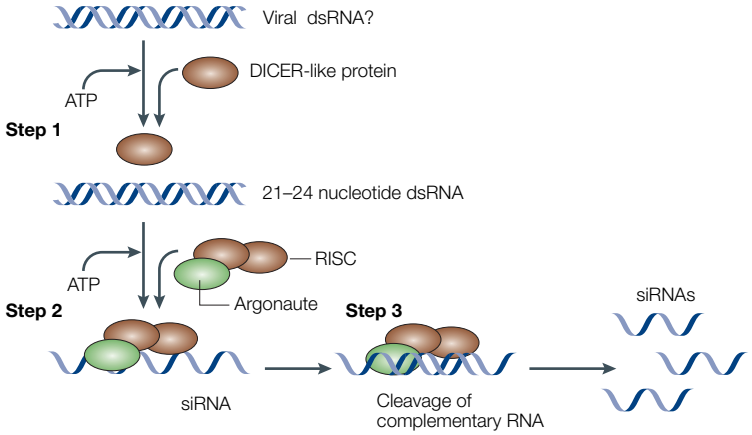
Mechanism of RNA silencing. Although the source of the trigger for RNA silencing is unidentified, much is known about the downstream events that silence viruses. Mutants that are defective in RNA silencing have been identified, facilitating the study of this mechanism. Plants have several homologues of the DICER endonuclease (BOX 2), and these DICER-LIKE (DCL) enzymes generate siRNA (short interfering RNA) in an antiviral response[79]. In *A. thaliana dcl1*, *dcl2* and *dcl3* mutants, viral siRNA accumulation upon infection with turnip mosaic virus (TuMV) and CMV is similar to that of wild-type plants[79]. However, in *dcl2* plants, accumulation of viral siRNA upon TCV infection is delayed, and these plants show enhanced susceptibility to TCV. Therefore, *DCL2* seems to be involved in the antiviral response to TCV infection.

Host RdRPs, which share no sequence homology with viral RdRPs, are components of the RNA silencing pathway[78] and seem to have a role in antiviral responses. The *A. thaliana* RdRP, RDR6, is required for transgene silencing and when mutated results in

Box 2 | RNA-silencing mechanism

Small RNA molecules (21–24 nucleotides long) that are found in multicellular eukaryotes function in development, chromatin modification, regulation of gene expression, protection of the genome against transposons and antiviral defence[96]. One class of small RNAs are named short interfering RNAs (siRNAs). They originate from precursors with long double-stranded RNA (dsRNA) pairing. siRNAs can be derived from genomically encoded repeated sequences, inverted repeats, transposons, retroelements or as products of cellular RNA-dependent RNA polymerase (RdRP) activity[73, 97]. They also arise from exogenous sources, such as viruses and transgenes that contain hairpin structures[98]. siRNAs guide the sequence-specific transcript degradation referred to as post-transcriptional gene silencing, and transcriptional silencing through chromatin modification[97]. A second class of small RNAs, microRNAs, are endogenously produced[99]. They are derived from long dsRNA hairpin structures and target several classes of proteins, particularly transcription factors that are involved in development[99].

Recently, the mechanism of RNA silencing has been characterized in plants, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Neurospora crassa*. Long precursor dsRNA is cleaved in an ATP-dependent step by the ribonuclease-III-type endonuclease DICER into 21–24-nucleotide duplexes (see the figure, step 1). In another ATP-dependent step, the siRNAs are unwound, probably by a DEAD-box RNA helicase, and become single-stranded. Next, these ribonucleoprotein complexes are rearranged into the RNA-induced silencing complex (RISC), which includes an Argonaute protein bound to the single-stranded siRNA (see the figure, step 2). The RISC binds and cleaves complementary target RNA in the middle of the paired region (see the figure, step 3)[73]. siRNAs then spread from cell to cell in the organism. In plants, it was hypothesized that the siRNAs move from cell to cell through plasmodesmata[100]. Members of the pumpkin PSRP1 class of proteins bind to single-stranded RNA species and mediate cell-to-cell movement[101]. siRNAs are transported in the phloem, and can trigger silencing throughout the plant[101].



hypersusceptibility to CMV[76,80]. Tobacco *NtRdRp1*-silenced plants were more susceptible to TMV than wild-type tobacco plants[81]. A PVX strain that does not usually spread in tobacco could spread both locally and systemically in transgenic plants lacking this inducible *NtRdRP1* (REF. 79). *N. benthamiana NbRdRp1m* is more than 90% identical to *NtRdRp1*, but encodes a truncated protein that does not function[82]. Interestingly, transgenic *N. benthamiana* expressing RdRP from *Medicago truncatula* showed an increased resistance to tobamoviruses, but not to other viruses[82]. The truncated RdRP might explain the hypersusceptiblity of *N. benthamiana* to viral infection[82]. The *A. thaliana RDR1*, which shares highest homology to *NtRdRp1*, is involved in, but not required for, viral-RNA silencing[83].

SYNERGISTIC INFECTION
An infection by two unrelated viruses during which one virus can replicate to higher-than-normal levels owing to the presence of the other virus.

Suppression of the antiviral pathway. Further support for RNA silencing as an antiviral mechanism comes from the evolution of viral proteins that suppress RNA silencing. These proteins inhibit different steps in the silencing pathway[84], indicating that convergent evolution of suppressors has occurred over time (TABLE 2). The generation of the silencing signal and the systemic spread of this signal are both targets of silencing suppressors. For instance, the structure of the tomato bushy stunt virus suppressor P19 has been solved and it binds tightly to double-stranded siRNAs, perhaps sequestering them from the RISC (RNA-induced silencing complex)[85,86]. Interestingly, P19 contacts the sugar-phosphate backbone of the siRNA and is therefore unaffected by its nucleotide composition[85,86]. P19 preferentially binds 20–22 nucleotide duplexes, and therefore only targets one class of small RNAs.

The primary function of suppressors is usually virulence. For example, the tobacco etch potyvirus encodes the proteinase HC-Pro (helper-competent proteinase), which is a virulence determinant[87] and also a suppressor of RNA silencing[88]. This function of HC-Pro as a silencing suppressor might explain the large number of SYNERGISTIC INFECTIONS involving potyviruses[88]. Many viruses that are normally unable to replicate and spread in a plant gain the ability to cause a systemic infection in the presence of tobacco etch potyvirus. Mutations in HC-Pro that abolish its suppressor function cause the virus to lose the ability to replicate and spread[89].

Several transgenic plants that constitutively express suppressors of viral-silencing have been generated[90–92]. Plants that express HC-Pro, P19 and P15 of the peanut clump virus have distinct morphological phenotypes that resemble *dcl-1* mutants[91,92]. Therefore, the virus suppressors seem to affect not only siRNA pathways, but also microRNA pathways (BOX 2). This effect on endogenous developmental pathways might explain some of the disease phenotypes.

RNA silencing and R gene signalling intersect. Both RNA silencing and *R* genes are antiviral defences. It seems probable that plants would develop a system that allows these pathways to communicate with each other to effectively limit viral infections. Is there crosstalk between these pathways? If so, which signalling molecules influence both pathways and could serve as links between the two? On the other hand, could viral suppressors of RNA silencing inhibit *R*-gene-mediated defences?

Evidence suggests that there is only limited crosstalk between these two important defence pathways. The same viral protein can function as a suppressor of RNA-silencing and as an Avr determinant in *R*-gene defence. TCV coat protein, the Avr determinant of *HRT*, is also a suppressor of RNA silencing[93]. The dual functions of this viral protein indicate that it is at the junction of these two antiviral pathways[94]. However, coat-protein mutants that do not interact with TIP (see above) and therefore cannot activate *HRT*-mediated defence are still functional suppressors of RNA silencing[94].

Table 2 | **Suppressors of RNA silencing encoded by plant viruses**

Virus	Suppressor	Virulence function	Suppressor function	Refs
African cassava mosaic virus	AC2	Virion sense gene expression, transactivator	ND	106
Barley stripe mosaic virus	γb	Seed transmission, virulence determinant, viral RNA and protein accumulation	ND	107
Beet necrotic yellow vein virus	P14	ND	ND	108
Beet western yellows virus	P0	Symptom determinant	ND	109
Beet yellow virus	P21	Enhances RNA accumulation	ND	110
Citrus tristeza virus	P20, P23, CP	P23-RNA-binding protein, regulates asymmetrical RNA accumulation, CP-virion assembly	CP and P20 suppress intercellular silencing. P20 and P23 suppress intracellular silencing	111–113
Cucumber mosaic virus	2b	Host-specific long-distance movement, inhibits salicylic-acid-mediated resistance	Blocks silencing signal from spreading	114,115
Peanut clump virus	P15	Viral accumulation	ND	108
Potato virus X	P25	RNA helicase, cell-to-cell movement	Blocks silencing signal from spreading	116
Rice hoja blanca virus	NS3	ND	ND	117
Rice yellow mottle virus	P1	Virus accumulation, long-distance movement, pathogenicity determinant	ND	106
Tobacco etch virus	HcPro	Aphid transmission, replication, systemic spread, polyprotein processing	Reverses silencing, blocks accumulation of short interfering RNAs	118
Tobacco mosaic virus	P126	Viral accumulation and movement	ND	119
Tobacco rattle virus	16K	Virus accumulation, seed transmission	ND	120
Tomato bushy stunt virus	P19	Symptom determinant, host-specific spread	Binds short interfering RNAs, reverses silencing, blocks silencing signal from spreading	106
Tomato mosaic virus	P130	Replicase	ND	121
Tomato spotted wilt virus	NSs	Viral virulence, putative movement protein	ND	117,122
Tomato yellow leaf curl virus	C2	Pathogenicity determinant	ND	123
Turnip crinkle virus	CP	Coat protein, movement	ND	93,124

ND, not determined.

Therefore, the function of TCV coat protein as an Avr determinant in *R*-gene-mediated defence is independent of its role as a suppressor of RNA silencing. However, one should consider that plants evolved the ability to detect Avr determinants, and that viruses did not evolve these molecules to activate defence, but as virulence factors. Perhaps plants targeted the suppressors for detection by another defence mechanism, the *R* genes.

It is possible that salicylic acid functions at the junction of RNA silencing and *R*-gene-mediated resistance. The RdRPs *NbRdRp1m*, *NtRdRp1* and *RDR1* are all inducible by both salicylic acid and certain viruses[81–83]. As the activation of many *R* genes results in the production of salicylic acid, *R*-gene-mediated resistance might cause the induction of these RdRPs. Although crosstalk might be limited, the biological concept of redundancy in defence responses might again be used by the plant to ensure survival.

Surprisingly, the silencing suppressor HC-Pro seems to enhance *R*-gene-mediated resistance. *N*-containing transgenic plants that express HC-Pro show increased resistance to TMV[95]. This raises the intriguing question of why viruses would evolve a seemingly counterproductive system of suppressing RNA silencing, only to enhance *R*-gene defence. Interestingly, when *HC-Pro N* transgenic plants are infected with several different viruses at elevated temperatures (which inhibit *N* function), increased infection symptoms are observed[87]. The symptoms are as severe as those observed during synergistic interactions involving potyviruses[87]. Therefore, in these experiments, HC-Pro functions to enhance viral virulence, a role that might be expected for a suppressor of antiviral defence. How does HC-Pro function in one situation to enhance antiviral responses and in the other to limit them? We eagerly await the results of studies involving other suppressors to help clarify this paradox.

Conclusions

There is functional overlap between *R*-gene-mediated resistance and RNA silencing. These are both ancient, conserved antiviral pathways, and understanding how they intersect and influence each other will shed light on disease-resistance mechanisms in plants. Many questions about both these defence mechanisms remain. One of the most important questions is how R proteins detect the presence of pathogen Avr components in the cell. It will be interesting to see what

the components of R-protein complexes are and how their dynamics alter in the presence of Avr proteins. Also to be addressed is the role of alternative splicing in TIR-NB-ARC-LRR function.

Similarly, many facets of RNA silencing as an antiviral defence are still open to investigation. One of the most interesting areas still to be addressed is the origin of the viral dsRNA trigger in plants. Whereas it seems that plants use the same RNA-silencing pathway for development and for defence, how this pathway evolved to have these dual essential functions is unknown, and it will be interesting to see if these two pathways can be separated. We will learn more about how RNA silencing operates as an antiviral mechanism as new suppressor functions are identified. By understanding how *R*-gene-mediated resistance and the RNA-silencing pathway intersect, it might be possible to manipulate resistance in plants.

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Competing interests statement
The authors declare no competing financial interests.

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