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Interaction between viral RNA silencing suppressors and host factors in plant immunity

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Short title

Zigzag model of the arms race between plants and viruses

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To elucidate events in the molecular arms race between the host and pathogen in evaluating plant immunity, a zigzag model is useful for uncovering aspects common to different host–pathogen interactions. By analogy of the steps in virus–host interactions with the steps in the standard zigzag model outlined in recent papers, we may regard RNA silencing as pattern-triggered immunity (PTI) against viruses, RNA silencing suppressors (RSSs) as effectors to overcome host RNA silencing and resistance gene (R-gene)-mediated defense as effector-triggered immunity (ETI) recognizing RSSs as avirulence proteins. However, because the standard zigzag model does not fully apply to some unique aspects in the interactions between a plant host and virus, we here defined a model especially designed for viruses. Although we simplified the phenomena involved in the virus–host interactions in the model, certain specific interactive steps can be explained by integrating additional host factors into the model. These host factors are thought to play an important role in maintaining the efficacy of

the various steps in the main pathway of defense against viruses in this model for virus–plant interactions. For example, we propose candidates that may interact with viral RSSs to induce the resistance response.

Introduction

Plants use two major strategies to defend against pathogens; the resistance (R)-protein-mediated strategy works effectively against diverse pathogens, including fungi, bacteria and viruses, while the RNA silencing strategy is a major antiviral mechanism [1-3]. Most viruses encode RNA silencing suppressors (RSSs) to interfere with RNA silencing [4,5]. As a consequence of the particular strategy used in the battle between virus and host, infected plants develop various symptoms [6].

According to the zigzag model (Figure 1A) to explain the two-branched immune system of plants in response to a plant pathogen [7•,8], R-protein-mediated resistance developed to control a pathogen that had overcome basal resistance or innate immunity, the first line of preformed, inducible defenses against the major groups of pathogens. Basal resistance starts with the detection of pathogen-associated molecular patterns (PAMPs), such as bacterial flagellin and fungal chitin, by the host's pattern-recognition receptors (PRRs). In the zigzag model, it is defined as pattern-triggered immunity (PTI). PRRs for bacteria and fungi have been identified, and these are mostly receptor-like kinases, which were once classified in the R-protein family. For example, the host transmembrane FLS2 protein recognizes the flg22 peptide from *Pseudomonas* flagellin [9]. To circumvent basal defense (PTI), pathogens produce effector proteins. When pathogen effectors overcame PTI, plants next evolutionally developed R-proteins to activate effector-triggered immunity (ETI), by which host proteins recognize the effectors as avirulence (Avr) factors, which then induces an amplified version of resistance comparable to PTI. R-protein-mediated resistance is often accompanied by a hypersensitive response (HR), which is observed as local

necrotic lesions. Therefore, we regard the HR-associated resistance response as a consequence of R-protein-mediated resistance, unless the HR pathway is independent of this resistance pathway.

There are not many comparative studies between antiviral and antibacterial/antifungal immune responses. Mandadi and Scholthof [10] have once reviewed analogous viral and nonviral immune concepts, but it was found not to be so simple to define viral PTI, ETI and ETS finding concrete examples; they did not actually integrate RNA silencing into their model. On the other hand, because RNA silencing against viruses is reminiscent of basal resistance against fungi and bacteria, by regarding RNA silencing as a type of PTI, viruses can be also integrated in a modified zigzag model; here, viral double-stranded RNA (dsRNA) corresponds to a PAMP [11,12]. However, there are certainly differences between viruses and other pathogens in their molecular interactions with plant hosts. In devising a model for viruses, we here integrate additional host factors to explain certain virus–host interactions and highlight aspects of the anti-viral defense that differ from the standard zigzag model for fungi and bacteria. In addition, we focus on the molecular cross-talk between RNA silencing and R-protein-mediated resistance. Figure 1C shows our entire scheme to explain the host–virus interactions in our model described here.

Comparison of the viral version of PTI and ETI with those in the standard zigzag model proposed for bacterial or fungal pathogens

To encompass all the phenomena involved in the complicated arms race between a particular host and pathogen, the zigzag model is quite useful. The concept of the model may be applied also to host–virus interactions, paying attention to some analogous phenomena in PTI and ETI. For example, one review totally fit a model for host defense against viruses to the standard zigzag model, regarding viral dsRNAs as PAMPs, host RNA silencing as PTI and counterattack by viral RSSs as ETS and so on

[12]. Consistent with this review, based on extreme resistance observed for tobacco plants expressing the P19 protein, an RSS of tombusvirus, Sansregret et al. [13•] showed that the general scheme of host induction and viral suppression of RNA silencing could be adapted to the classical frame of PTI and ETI. However, in another review, although some degree of analogy of PTI and ETI between viruses and other pathogens was drawn, the author indicated a clear difference and the uniqueness in virus–host interactions [11,14]. Fungal and bacterial pathogens have various Avr proteins in their arsenal when ETI is activated; one can be replaced by other redundant effectors. However, viruses have a limited number of proteins that are all important for their survival. When one of the viral proteins is recognized by a host R protein, viruses cannot easily replace it with another; rather they modify it by changing the amino acid sequence while retaining the protein structure necessary for the function. Whether the host R protein still recognizes the modified version depends on the LRR domain in the R protein with a varying degree of affinity. Alternatively, according to the bait and switch model, a host co-factor that binds to an R protein may affect the specificity of the host recognition for the viral Avr protein [10]. Therefore, for virus–host interactions, we cannot draw an actual zigzag model in which multiple rounds of ETS followed by ETI are repeated with different combinations of host R protein and viral Avr. As such, the molecular virus–host interaction must be explained by a limited number of players.

Although the idea that RNA silencing and its suppression by viral RSSs can be rationalized within the PTI–ETI framework is attractive, we need more experimental evidence because there are actually viral proteins that are not RSSs but are recognized by R-proteins. Instead of expanding on the standard zigzag model to fit virus–host interactions, we can create a model that allows a quick overview of the molecular phenomena in the virus–host arms race, the strategies unique to viruses and the steps that are analogous to the standard zigzag model. As we will discuss, we consider that

the host response branches from the general course of antiviral response instead of repeating the ETI; the strategies at these branches vary depending on the specific host and virus and the particular point of the interaction.

RNA silencing and viral RNA silencing suppressors

RNA silencing functions as an antiviral mechanism in plants [2,4] (Figure 1, B-D). As a counterdefense, viruses developed RNA silencing suppressor (RSS) proteins, which function to inhibit RNA silencing through diverse modes of action. The main mechanism for the RSSs appears to be binding with long dsRNA or siRNA duplexes, subsequently inhibiting siRNA biogenesis or RISC formation [15]. Another mechanism is binding to the components in the silencing pathway such as AGO1. Several RSSs (TCV CP, CMV 2b, TBSV P19, PVX P25, Polerovirus P0 and P1 of *Sweet potato mild mottle virus*) have been reported to repress or interfere with the function of AGO1 [16-21]. Diverse RSSs appear to reduce AGO1 in infected plant tissues [22]. However, although viral RSSs interfere with host RNA silencing and are mostly effective, hosts have some mechanisms to activate another or secondary defense. For example, *Arabidopsis thaliana* encodes 10 AGO proteins. AGO1 performs not only antiviral RNA silencing, but also silences endogenous genes by cleaving viral RNA and endogenous target mRNA. Recent screening of other AGO proteins in antiviral defense using knockout mutants revealed that AGO2 is also induced and functions in the defense against TCV and CMV when the viral RSSs targeted AGO1 [23]. This study suggested that AGO2 is involved in antiviral RNA silencing, which is induced via infection by viruses that encode RSSs targeting AGO1 or via miRNA-mediated RNA silencing because AGO2 expression is repressed by AGO1 via miR403. In addition, miRNA-mediated RNA silencing appears to control other RNA silencing components, including DCLs, DRB4, RDR6, and AGOs [24], implying their involvement in secondary antiviral RNA silencing. Interestingly, AGO2

is also involved in the induction and secretion of antimicrobial pathogenesis-related protein 1, in addition to antiviral RNA silencing [25]. Since DRB4 is involved not only in RNA silencing but also in R-gene-mediated resistance, if many R-genes are controlled by miRNAs, seemingly, when a virus suppresses RNA silencing, diverse secondary defense systems could be activated.

Viral RNA silencing suppressors as Avr determinants

Direct and indirect interactions occur between R-gene-mediated resistance and RSSs. For example, a link between ETI-like phase and RNA silencing has been suggested for *Cucumber mosaic virus* (CMV), *Tobacco etch virus* (TEV), and *Potato virus Y* (PVY). The RSS of CMV, the 2b protein (CMV 2b), inhibits the salicylic acid (SA)-mediated defense response [26]. Some examples of molecular interactions have been reported between a viral RSS and an R-protein [27-29]. Well-established examples of host recognition of an RSS are the coat protein (CP) of *Turnip crinkle virus* (TCV) and the replicase of *Tobacco mosaic virus* (TMV). The TCV CP serves as an RSS, but also as the TCV Avr protein that induces R-gene (the *HRT* gene)-mediated resistance in *Arabidopsis* ecotype Di-17 [30,31]. TMV replicase has RSS activity [32], and the p50 helicase domain in the replicase can induce an HR, serving as the Avr determinant in tobacco carrying the N-gene, which is the well-known R-gene working for ETI against TMV.

Using an agroinfiltration assay to study the ability of viral RSSs to elicit HR-like necrosis, Angel and coworkers [33] also found that the P19 protein (P19) of *Tomato bushy stunt virus* (TBSV) was recognized by a putative R-protein in *Nicotiana* species, which then induced an HR-like necrosis. Using a similar agroinfiltration assay for *Capsicum annuum*, Ronde and colleagues [34] recently showed that the RSS of *Tomato spotted wilt virus* (TSWV), the NSs protein, function as the Avr determinant in

C. annuum carrying the R-gene (*Tsw*) against TSWV. They discussed their pathosystem in light of the putative interplay between RNA silencing and the R-gene-mediated resistance.

Consistent with the reports by Angel and colleagues [33,35], P19 was recently demonstrated to function as the Avr protein that induced extreme resistance (ER), characterized by strong SA-dependent resistance without visible HR lesions, in *Nicotiana tabacum* [13•]. In addition, the binding of P19 to small RNA (sRNA) was necessary to induce ER, suggesting that RNA silencing and an ETI-like phase are linked to each other. Similarly, the 2b protein (TAV 2b) of *Tomato aspermy virus* (TAV), an RSS of TAV, was found to induce HR on the leaves of *N. tabacum* and *Nicotiana benthamiana* infected with the TMV vector expressing TAV 2b [36]. In this case, Lys 21 and Arg 28, both located within the N-terminal region of TAV 2b, were critical for the HR induction. These positively charged residues were later shown to be involved in sRNA binding and thus the RSS activity of TAV 2b [37]. These studies thus suggest the existence of an R-protein that recognizes TAV 2b with RSS activity as an Avr protein in *Nicotiana* species. However, when its expression was driven by its own parent virus or when 2b of CMV, which is closely related to TAV, was expressed by the TMV vector, no necrotic lesions were observed [36]. These contradictory observations imply additional involvement of other viral or host factors in the HR-associated resistance responses in specific combinations of RSS and host.

In further support of 2b serving as an Avr protein, we recently demonstrated that CMV 2b induced weak necrosis and SA and hydrogen peroxide accumulation in *Arabidopsis thaliana* Col-0 ecotype (hereafter, *Arabidopsis*), suggesting that the plant has an R-protein that recognizes CMV 2b as an Avr protein. In fact, CMV Y strain (CMV-Y) causes mosaics with fine necrotic spots in the upper leaves, but not typical HR-like

necrosis, although we observed slightly stronger necrosis on Col-0 infected with CMV-HL (a lily strain). From the results of an in situ molecular interaction study, the necrosis on *Arabidopsis* seemed to have been driven by a specific interaction between CMV 2b and the *Arabidopsis* catalase-3 (CAT3) [38••,39], a key enzyme in cellular scavenging of hydrogen peroxide and induction of HR. If this type of HR-like induction is indeed part of the host ETI-like phase, then an *Arabidopsis* R-protein may recognize CMV 2b as a complex with a host factor(s) that includes CAT3. The affinity between CMV 2b and CAT3 seems to be important for determining the degree of necrosis because the observed necrosis depends on the CMV strain and the *Arabidopsis* ecotype.

miRNA-mediated regulation of the R-genes against viruses

Recent studies demonstrated that plant microRNAs (miRNAs) target and negatively regulate R-gene expression via RNA silencing [29-33]. This miRNA-mediated R-gene regulation was actually inhibited upon viral infection, suggesting that RNA silencing is linked to R-protein-mediated resistance. Downregulation of R-gene expression by RNA silencing is perhaps because plants prevent unwanted autoimmunity by overexpressing the R-gene in the absence of viruses. Although RNA silencing primarily targets viruses in the model, we also consider that RNA silencing can also affect the subsequent host defense governed by R-proteins as discussed here.

Recent evidence has indicated that plant small RNAs (sRNAs) (siRNAs and miRNAs) are involved in the basal resistance against pathogens. For bacteria, the bacterial peptide flag22 actually induces miRNA393, which targets auxin receptors, which in turn mediate the signaling that activates the SA resistance pathway [40]. In fact, an RNA-induced silencing complex (RISC) containing Argonaute 2 (AGO2) programmed with miR393 plays a critical role in ETI against *Pseudomonas syringae* [25]. The

suppression of auxin signaling by miR393 ultimately activates the SA-mediated defense response, which is also one of the main mechanisms in the antiviral PTI-like phase; interference with the miR393-mediated regulation of auxin receptors by viral suppressors must impair the PTI- and ETI-like phases against viruses.

Some sRNA can target R-gene transcripts directly. With the recent discovery of many new miRNAs through deep-sequencing studies (e.g., RNASeq),. bioinformatics analyses of the sRNA libraries obtained have identified novel miRNAs and putative functions that potentially target host R-genes. For example, He and colleagues [41] found an miRNA in *Brassica rapa* (named bra-miR1885) that was induced by *Turnip mosaic virus* (TuMV) infection and potentially targeted the mRNAs of R-genes encoding TIR-NB-LRR class proteins; unfortunately, whether the R-gene targets are involved in the host resistance to TuMV was not determined. As another example, after searching tobacco sRNA libraries for N-gene-related sRNAs, Li and coworkers [42•] found that two newly discovered plant miRNAs (nta-miR6019 and nta-miR6020) could guide cleavage of the N-gene transcript in tobacco, conferring resistance to TMV. In a search for phased, *trans*-acting siRNAs (phasiRNAs) isolated from *Medicago* after deep sequencing, Zhai and colleagues [43] revealed that the majority of phasiRNAs were produced from R-genes, suggesting a close association between RNA silencing and the R-gene-mediated resistance response. Although the phasiRNAs targets have not been identified, we should consider generation of phasiRNAs when trying to understand R-gene-mediated immunity.

Host factor(s) that regulate the interactions between RNA silencing and R-gene-mediated resistance in a model for viruses

In our model for viruses (Figure 1B-D), viruses first produce dsRNAs in infected plants. In turn, plants activate RNA silencing as a PTI-like phase to target the viral RNAs. Then, the viruses produce RSSs as viral effectors to suppress RNA silencing. In

the subsequent ETI-like phase, to generate an effective defense, the plants should activate an R-protein that specifically recognizes the viral RSSs as the Avr protein, thus leading to the HR and SA-dependent resistance. Although we have simplified each phase to give a general overview, depending on the particular host–virus combination, additional host factors should be integrated into the model for understanding certain specific stages in host–virus interactions. For example, the RNA silencing component DRB4 is potentially one such mediator of PTI-like phase. DRB4 is a dsRNA-binding protein that associates with a dicer-like protein 4 (DCL4) to produce virus-specific siRNA [44,45]. A recent study revealed that the *Arabidopsis* R-protein requires DRB4 for the subsequent HRT (an R protein to TCV)-mediated HR against the RSS or CP of TCV [46••]. Notably, DRB4 interacts with both HRT and the TCV CP and stabilizes HRT, but inhibits the interaction between HRT and the TCV CP. Although we do not yet know how DRB4 contributes to the HR, we do know that DRB4 is also involved in R-gene-mediated resistance against bacteria, implying that it is involved in ETI. Another candidate mediator is the plant calmodulin-like protein, rgs-CaM, which we describe in detail next.

Possible branches in the model between plants and viruses

RSSs that can suppress SA-related defense responses include CMV 2b, CaMV P6, and TCV CP [14•,26,47-49]. Since viral RSSs participate in an arms race between viruses and plants, and since RNA silencing is a PTI-like phase against diverse viruses, RSSs reduce host defense, shifting the phase to effector-triggered susceptibility (ETS)-like in the model. On the other hand, because some RSSs also behave as an SA-mediated immunity suppressor (SIS), those RSSs can be considered to create another ETS-like phase, implying additional branches in the model. All of these RSSs also function as avirulence proteins, which can elicit the HR in plants that possess the corresponding R-gene. In these cases, the HR is closely associated with SA-related defense responses.

Integrating all these data, we propose a model in which viral SIS has been developed to repress or evade the HR-mediated resistance against viruses (Figure 1, B and D). If a virus has RSS or other viral proteins with SIS ability, SIS might be able to mask the R-protein-mediated defense responses, resulting in a phenotype similar to that seen in a susceptible plant. Therefore, we believe that many potential resistant interactions between viruses and plants are still hidden. For example, the exacerbation of the HR and symptoms that accompanies necrosis in plants infected with virus vectors that express heterologous viral RSSs or other proteins [36,50] might be explained by the induction of defense responses to the expressed viral proteins, which are not induced by the parental viruses because of the viral SISs.

In addition to R-gene-mediated resistance, recent studies have suggested that plants have additional counter-counterdefense systems against viral RSSs. We discovered an antiviral counter-counterdefense that involves rgs-CaM in tobacco [51••,52]. When rgs-CaM was initially found, rgs-CaM was reported to interact with the TEV RSS, HC-Pro and act as an endogenous RSS [53,54]. Later, we found another function for rgs-CaM in antiviral defense. Our previous study suggested that rgs-CaM binds not only HC-Pro, but also other RSSs, including CMV and TAV 2b, via its affinity to the negatively charged dsRNA-binding domains of RSSs. Then, rgs-CaM presumably reinforces antiviral RNA silencing by directing the degradation of its associated RSSs via autophagy (Figure 1, C and D). Calmodulin-like proteins are one of the three protein families of EF-hand Ca^{2+} sensors in plants and are thought to coordinate the functions of several endogenous proteins by binding to the targets as a hub protein in response to the Ca^{2+} stimulus [55]. Since they are known to function in countering abiotic and biotic stresses, we suspect that rgs-CaM functions in antiviral defense [41].

Recent studies on the interaction of the TCV CP with the *Arabidopsis* NAC transcription factor (TIP) imply that an alternative branch should be included in the model between viruses and plants. TCV CP is a viral RSS [56,57] and the avirulence protein recognized by the R-gene, HRT in *Arabidopsis* Di-17 [31], suggesting that TIP is involved in the RSS activity and HR induction by the TCV CP. However, TIP is not required for either the RSS activity or HR induction. Instead, TIP was recently shown to be involved in SA-mediated basal immunity in *Arabidopsis* [58]. TCV CP suppresses the SA-mediated basal immunity via its binding to TIP [14•]. These studies indicate that TCV CP suppresses both the RNA silencing and SA-mediated basal immunity to facilitate the initial infection of *Arabidopsis* with TCV (Figure 1D). In the canonical zigzag model for the interaction between other microorganisms and plants, PTI should be an induced defense, which partly shares defense responses with ETI after the perception of PAMPs by receptor-like kinases. Therefore, the TIP-associated PTI seems to be integrated in the viral model as another interaction between virus and host. Here, we draw those new interactions as branches in the viral model. More interestingly, the basal resistance involving TIP also affects CMV accumulation, indicating that the basal resistance is not specific to TCV [58]. For many other viruses, similar sets of defense-related genes have been reported to be induced during viral infection of a susceptible plant [59,60], suggesting that the host resistance response is somehow suppressed in those plants, although it is partly activated. As such interactions are uncovered, we can better organize the branches from the main course of defense involving RNA silencing in the model for host–virus interactions.

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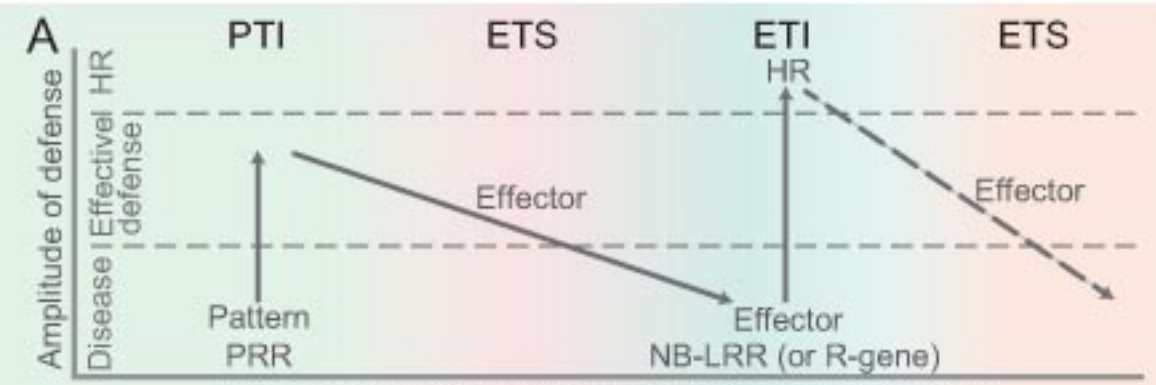
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Figure legend

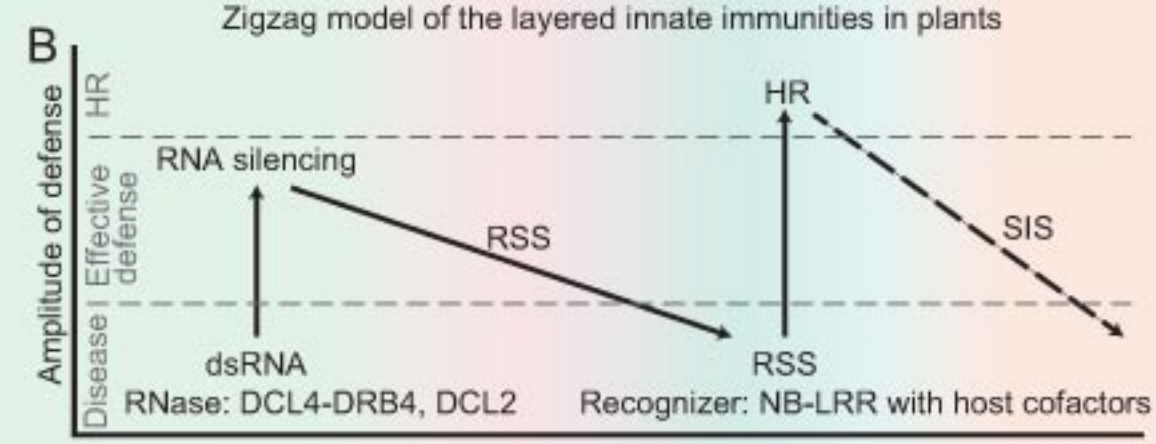
Figure 1

The interactions between viral RSS (SIS) and host factors involved in plant immunity. **(A)** Model of the arms race between pathogens and plants using the standard zigzag model. **(B)** Model of molecular virus–host interactions involving RNA silencing and R-protein (NB-LRR)-mediated resistance. Unlike the innate immunity against other pathogens, the first layer of the immunity against viruses is RNA silencing. RNA silencing is induced by intra- and intermolecularly formed double-stranded RNAs (dsRNAs) of the viral genome or its transcripts. Then, dsRNA is processed into siRNAs by the DCL4–DRB4 complex and DCL2 in *Arabidopsis*. AGO1 binds siRNA and cleaves viral RNA guided by the incorporated siRNA. Most viruses counteract this by expressing RNA silencing suppressors (RSSs). Plants coevolved an immune system that is associated with the HR in response to the RSS. In this figure, the HR that is not associated with SA-mediated resistance is defined as programmed cell death (PCD). Recent studies have suggested that host cofactors such as DRB4, a tobacco calmodulin-like protein, rgs-CaM, and the *Arabidopsis* NAC transcription factor (TIP) help putative NB-LRRs to recognize RSSs [46••]. Salicylic-acid (SA)-mediated defense responses were found to be suppressed by RSSs such as CMV 2b and TCV CP, suggesting viral evasion of induced HR, which is associated with the SA-mediated immunity to prevent viral infection. Here, an SA-mediated immunity suppressor is designated SIS. **(C)** Entire scheme to explain the host–virus interactions, integrating steps unique to viruses compared with the standard zigzag model. **(D)** Branches from the main path of the model, where viral factors (RSS and SIS) participate, represent other virus–host interactions that are mediated by the same viral factors. For example, the SA-mediated basal immunity involving TIP [58] and the rgs-CaM-directed

559 degradation of RSS via autophagy [51••] are also thought to contribute to antiviral
560 immunity, although TIP and rgs-Cam seem to be independent of the general course of
561 host defense. TCV CP counteractively suppresses the basal immunity by binding to
562 TIP [14•].
563



Zigzag model of the layered innate immunities in plants



Main scheme of arms race between viruses and plants

