Extreme Resistance as a Host Counter-counter Defense against Viral Suppression of RNA Silencing

Raphaël Sansregret¹, Vanessa Dufour¹, Mathieu Langlois¹, Fouad Daayf², Patrice Dunoyer³, Olivier Voinnet³,⁴*, Kamal Bouarab¹*

¹Centre SEVE, Département de Biologie, Faculté des Sciences, Université de Sherbrooke, Sherbrooke, Quebec, Canada, ²Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada, ³Institut de Biologie Moléculaire des Plantes du CNRS, Université de Strasbourg, Strasbourg, France, ⁴Department of Biology, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland

Abstract

RNA silencing mediated by small RNAs (sRNAs) is a conserved regulatory process with key antiviral and antimicrobial roles in eukaryotes. A widespread counter-defense strategy of viruses against RNA silencing is to deploy viral suppressors of RNA silencing (VSRs), epitomized by the P19 protein of tombusviruses, which sequesters sRNAs and compromises their downstream action. Here, we provide evidence that specific Nicotiana species are able to sense and, in turn, antagonize the effects of P19 by activating a highly potent immune response that protects tissues against Tomato bushy stunt virus infection. This immunity is salicylate- and ethylene-dependent, and occurs without microscopic cell death, providing an example of “extreme resistance” (ER). We show that the capacity of P19 to bind sRNA, which is mandatory for its VSR function, is also necessary to induce ER, and that effects downstream of P19-sRNA complex formation are the likely determinants of the induced resistance. Accordingly, VSRs unrelated to P19 that also bind sRNA compromise the onset of P19-elicited defense, but do not alter a resistance phenotype conferred by a viral protein without VSR activity. These results show that plants have evolved specific responses against the damages incurred by VSRs to the cellular silencing machinery, a likely necessary step in the never-ending molecular arms race opposing pathogens to their hosts.

Citation: Sansregret R, Dufour V, Langlois M, Daayf F, Dunoyer P, et al. (2013) Extreme Resistance as a Host Counter-counter Defense against Viral Suppression of RNA Silencing. PLoS Pathog 9(6): e1003435. doi:10.1371/journal.ppat.1003435

Editor: Shou-Wei Ding, University of California Riverside, United States of America

Received September 25, 2012; Accepted May 6, 2013; Published June 13, 2013

Copyright: © 2013 Sansregret et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: RS is a fellow of the FQRNT. KB and FD are supported by Discovery grants from the Natural Sciences and Engineering Research Council of Canada (NSERC). OV was supported by an award from the Bettencourt Foundation and the Louis D. Prize from the French Academy of Science. PD was supported by the Agence National pour la recherche (ANR-08-JCJC-0063 and ANR-10-LABX-36). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: voinnet@ethz.ch (OV); Kamal.Bouarab@USherbrooke.ca (KB)

Introduction

Plants fight microbial attacks using both constitutive and induced defenses, which include basal and highly specific resistance [1]. Basal resistance, or PTI (for PAMP-Triggered Immunity), often relies on the detection of highly conserved signature molecules that include fungal polysaccharides or bacterial flagellin, collectively termed pathogen-associated molecular patterns (PAMPs; [1,2]). To circumvent this first layer of defense, many host-adapted microbes produce effector proteins that suppress various steps of PTI [3]. As a counter-response, plants have, in turn, evolved classes of specialized receptors called resistance (R) proteins that directly detect pathogen’s encoded suppressors of PTI, or that sense the molecular consequences of their adverse action on defense-related host factors.

R protein activation triggers potent defense responses collectively named Effector Triggered Immunity (ETI) that often altogether not always (see below) culminate in Hypersensitive Response (HR), a rapid and localized cell death process thought to limit or preclude pathogens’ growth [1,2]. As a consequence of the gene-for-gene type of interaction linking these two components, plant R genes and their corresponding pathogen-encoded virulence factors evolve constantly and rapidly, so that HR, a common and ultimate manifestation of ETI, is usually only observed in specific plant species infected with specific pathogen strains. The plant hormones salicylic acid (SA), ethylene and jasmonic acid (JA) are crucially implicated in signaling networks underpinning both PTI and ETI [1,2,4,5]; antimicrobial pathogenesis-Related Proteins (PRs), which include taumatine-like proteins and chitinases, are also often induced by both pathways and constitute, therefore, typical molecular markers of pathogen-induced defenses [6]. Although the occurrence of HR is classically used to discern PTI from ETI during bacterial or fungal infections [7], an HR-independent process known as Extreme Resistance (ER) is activated by a number of R proteins during ETI against viruses; ER is characterized by the lack of detectable accumulation of the triggering virus, and is accompanied by the onset of a broad-spectrum antiviral state in the absence of macroscopic or microscopic cell death lesions [8–11].

RNA silencing is a conserved regulatory process that has evolved as an antiviral and antimicrobial defense mechanism in plants and animals [12–17]. Common features of RNA silencing across organisms include the involvement of double-stranded (ds)RNA as an initiator molecule, and accumulation of 21–24 nt small (s)RNAs that are processed from dsRNA by the RNAse III-like enzyme Dicer [18–20]. sRNAs are then incorporated into Argonaute (AGO)-containing effector complexes termed RNA-induced Silencing Complexes (RISCs) and, in case of extensive
Multiple and complex layers of defense help plants to combat pathogens. A first line of defense relies on the detection, via dedicated host-encoded receptors, of signature molecules (so-called pathogen-associated molecular patterns, PAMPs) produced by pathogens. In turn, this PAMP-triggered immunity (PTI) may be itself antagonized by adapted pathogens that have evolved virulence effectors to target key PTI components. Host plants react to PTI suppression by producing disease resistance (R) proteins that recognize virulence effectors and activate highly specific resistance called Effector Triggered Immunity (ETI). It has been noted that RNA silencing, a sequence-specific antiviral defense response based on the production of virus-derived 21–24 nt small RNAs on the one hand, and its suppression by virulence effectors, called viral suppressors of RNA silencing (VSRs) on the other, are conceptually similar to PTI. Here we provide strong support to this hypothesis by showing that extreme resistance is indeed activated following detection, in specific host species, of the VSR activity of a viral virulence effector. The ensuing antiviral immunity displays many characteristics of ETI, suggesting that one or several R proteins must sense the integrity of the host silencing machinery.

Sequence complementarity between sRNA guide and target, AGO catalyzes cleavage of the target RNA. Arabidopsis thaliana possesses four Dicer-like (DCLs) and ten AGO proteins [21], among which DCL4 and its surrogate DCL2, as well as AGO1 and AGO2, play essential roles as processors and effectors of virus-derived short interfering (si)RNAs, respectively [22–29]. DCL1- and AGO1-dependent micro (mi)RNAs produced from endogenous loci regulate the expression of many transcripts displaying miRNA sequence-complementarity, including mRNAs for transcription factors, enzymes, and regulators of PTI induced, notably, by bacteria [15–17,30,31].

As a consequence of these multiple RNA silencing-based defense layers, plant viruses, pathogenic bacteria, oomycetes and, possibly, fungi, have evolved suppressors of RNA silencing (SRs) that apparently target many steps of the siRNA and miRNA pathways [14,32–37]. SRs are highly diverse in sequence, structure, and activity, and single SRs may target multiple points in RNA silencing pathways [14,31]. Several viral SRs (VSRs) are known to affect AGO1 function [14]. For example, the Beet western yellow virus P0 protein was suggested to act as an F-box protein targeting AGO proteins for degradation, thereby preventing RISC assembly [38–40]. Turnip crinkle virus P38 was recently shown to bind directly and specifically AGO1 through mimicry of host-encoded glycine/tryptophane (GW)-containing proteins normally required for RISC assembly/function in diverse organisms [41,42]. Physical sequestration of siRNAs is another common property of VSRs miRNA [43–47], although the extent to which this specific feature contributes to effective RNA silencing suppression in vivo remains unclear [42]. The most compelling example of active silencing suppression mediated by siRNA binding is provided by the tombusvirus P19 protein, of which the closely related Tomato bushy stunt virus (TBSV) and Carnation Bacterioid ringspot virus (CIRV; 97% identity) are the type representatives. Following its original discovery as a VSR [33], P19 was co-crystallized as a head-to-tail homodimer in direct association with an siRNA duplex [43,48]. Supporting a direct and critical contribution of homodimerization and siRNA binding to the P19 VSR activity, stable point mutants of the proteins lacking either property display complete loss-of-VSR-function phenotypes in both virus-infected and transgenic plants [43,49–51]. siRNA binding by P19 also explains why its constitutive expression in Arabidopsis promotes developmental defects resembling those of plants carrying mutations in miRNA pathway components. Indeed, it was shown that P19 binds endogenous siRNAs and miRNAs, incurring, in the process, misregulation of the cognate endogenous targets of these molecules [42,52].

Remarkable parallels can be drawn between the general framework of silencing activation and its suppression by pathogens on one hand, and the classical PTI-ETI scheme for resistance, on the other. This has prompted the suggestion that the two processes might be, in fact, manifestations of similar, if not identical, phenomena [31,53]. In the case of (+)-stranded RNA viruses, for example, viral-derived dsRNA can be assimilated to a PAMP because this molecule is a mandatory product of viral replication. Similarly, the Dicer/AGO consortium orchestrating the antiviral reaction may be conceptually compared to the first defense layer underlying PTI [31]. Pursuing the comparison one step further and taking into account that VSRs are virulence effectors, it can be anticipated that the damages incurred by VSRs to the cellular silencing machinery may be sensed by host-encoded functions comprising, perhaps, dedicated R genes; the effects of such functions would thus be diagnosed, at least partly, by the typical outputs of ETI, including HR [31]. Supporting this notion, at least three VSR proteins from distinct virus families are known to trigger HR-like lesions in a host-specific manner [53–56]. It remains largely unknown, however, if these responses are stimulated by intrinsic silencing suppression properties or by other, unrelated functions of the viral proteins involved. Also unclear is whether virus resistance is effectively triggered upon recognition of these VSRs in these specific hosts, and to what extent the output of the induced defense compares with that of classical ETI.

The present series of experiments was aimed at addressing these various issues using the well-characterized P19 VSR in tobacco. The results support the idea that RNA silencing and its suppression by viruses can be effectively rationalized within the frame of PTI-ETI, since we demonstrate, in authentic infection contexts, that (i) tombusviral virulence (ii) suppression of RNA silencing and (iii) induction of an ER-type of resistance with molecular features of ETI are all dependent upon the ability of P19 to bind sRNAs. Collectively, the data support the existence of host-encoded sensors that monitor the status/integrity of key RNA silencing components in plants. We propose, consequently, that perturbation of these components by pathogen-encoded SRs may activate potent ETI-like resistance responses. This proposed host counter-counter defensive layer likely constitutes an important driver in the evolution and diversification of SRs from viruses and perhaps other parasites.

**Results**

**P19 is required to trigger an ETI-like resistance against TBSV in N. tabacum**

TBSV P19 was shown to induce a HR-like response in N. tabacum and other Nicotiana species; a host-specific response strongly evocative of R gene-mediated ETI [34–57]. To ascertain further if, indeed, P19 acts as an elicitor of immune responses, we generated transgenic N. tabacum cv. Xanthi lines expressing P19 under the GVG glucocorticoid inducible promoter, which is activated by dexamathazone (Dex::P19) [59]. The expression of P19 was quantified in two independent lines 0, 12 and 24 hours post Dex application (hpp); non-transgenic plants sprayed with...
DEX provided a negative control. While very low P19 transcript accumulation was observed before DEX treatment in the two transgenic lines, it was up to 4000 times higher following DEX application, at 12 and 24 hpp, compared to 0 hpp and to DEX-treated non-transgenic plants (Figure 1A). Accumulation of the P19 protein, mostly under homodimeric form, was also detected by Western analysis in the DEX-induced transgenic lines, but not in non-transgenic lines, using a polyclonal P19 antibody (Figure 1B). Accumulation of P19 following DEX induction correlated with the onset of three key markers of plant defense responses: (i) the progressive development of HR-like lesions in the sprayed areas of leaves, (ii) the accumulation of distinct PR proteins, PR1, PR2 and PR3, at 24 and 48 hpp (Figures 1C–D), and (iii) the accumulation of salicylic acid (SA) which was 4–5 times higher following DEX application at 24 hpp in the DEX-induced transgenic lines compared to 0 hpp and to DEX-treated non-transgenic plants (Figure S1). Collectively, therefore, the results presented in Figure 1 and Figure S1 suggest that in N. tabacum, P19 effectively acts as an elicitor of plant defense responses displaying at least superficial characteristics of ETI.

To test if P19 effectively induces resistance against TBSV in N. tabacum, Agrobacterium strains expressing either TBSV-GFP or TBSVAP19-GFP, which is unable to express P19 [26], were used to inoculate leaves of 5-week old N. tabacum. At 5 days post-infiltration (dpi), virus accumulation was monitored under UV light via the appearance of green fluorescence in infiltrated leaves, and by Western analysis using an anti-GFP antibody. Viral replication was assessed directly in parallel by Northern analysis, using a GFP DNA fragment as a probe, which detects both genomic and sub-genomic RNAs of TBSV-GFP. We found that the presence or absence of P19 expression from TBSV-GFP had dramatically contrasted consequences on virus replication. Thus, GFP was not observed (Figure 2A–B) and the viral RNAs were below detection limits of Northern analyses (Figure 2C) in TBSV-GFP-inoculated leaves. In sharp contrast, however, both GFP accumulation and viral RNA replication were readily detectable in TBSVAP19-GFP-infiltrated leaves at 5 dpi (Figures 2A–C). To further characterize the P19-mediated defense response, we used trypan blue staining as a diagnostic of cell death. Leaves were thus inoculated either with TBSV-GFP, P50 from Tobacco mosaic virus

![Image](image-url)
(TMV), which induces an HR in *N. tabacum* carrying the resistance gene N (as a positive control), or GUS as a negative control. We found that the visible and microscopic HR observed in P50-treated plants was absent from TBSV-GFP-infected and control leaves (Figure S2). These results strongly suggest that extreme resistance (ER) was triggered in TBSV-GFP-inoculated leaves of *N. tabacum*, and implicate, therefore, P19 as the elicitor of this defense. In fact, the results obtained here with P19 in tobacco are highly reminiscent of the well-studied interaction between *Potato virus X* coat protein (CP) and the Rx resistance protein in *Solanum tuberosum* or tobacco [8]. Indeed, while Rx typically confers ER to PVX in the context of authentic virus infections, isolated and prolonged production of CP, for instance via Agrobacterium-mediated transient expression, does trigger an HR in Rx potato genotypes [8,9], as seen previously and here upon transient and transgenic expression of P19 in specific *Nicotiana* species (Figure 1B–C, [56]). With both PVX and TBSV, the potent antiviral state accompanying the ER (e.g. Figure 2C) probably stops virus replication before the CP or P19 have reached the levels required to trigger an HR [8,9], a phenomenon presumably bypassed when both elicitors are produced in a virus replication-independent manner.

**Salicylic acid and ethylene are required for extreme resistance induced by P19**

The potent (Figure 2C) and broad-spectrum [8] antiviral state triggered by ER is suspected to underlie the production of defense-related hormones, including SA, which possesses demonstrated antiviral activities [27,60,61]. The gaseous hormone ethylene is also important for induction of plant immunity [4]. To investigate the possible roles of these compounds in the ER-like resistance induced by P19 against TBSV, SA-deficient transgenic tobacco plants expressing NahG (Salicylate hydroxylase; [62]) and plants insensitive to Ethylene (ETR; [63]) were inoculated with TBSV-GFP using Agrobacterium-mediated delivery. At 5 dpi, leaves were observed under UV and samples were harvested for Western analysis using the anti-GFP antibody. Unlike WT plants, both transgenic plants failed to display resistance against TBSV (Figure 3A–B) and, accordingly, the P19-dependent induction of PR proteins was compromised in NahG plants ([64,65]; Figure 2).

**Figure 2. P19 is required for extreme resistance of *N. tabacum* against TBSV.** (A) Leaves of 5 weeks old *N. tabacum* cv. Xanthi plants were infiltrated with Agrobacterium expressing TBSV-GFP or TBSVAP19-GFP. Pictures of infiltrated leaves were taken 5 dpi under transmitted light and UV. (B) GFP accumulation in infiltrated leaves from three independent plants. Western analysis was carried out using an anti-GFP antibody. Coomassie staining of the same extracts is shown to demonstrate equal protein loading. (C) Northern analysis of TBSV-GFP and TBSVAP19-GFP RNA accumulation in infected plants at 5 dpi, using a GFP DNA fragment as a radioactive probe. Viral genomic and subgenomic RNAs are indicated; ribosomal RNA was used to demonstrate equal RNA loading. Experiments were repeated three times and showed similar results. doi:10.1371/journal.ppat.1003435.g002

Suppressor of RNA Silencing and Extreme Resistance
S3). Overall, these results indicate that SA and ethylene are required for the ER induced by P19 against TBSV. We then investigated if the HR-like lesions induced by P19 in tobacco leaves (Figure 1C) were SA- and/or ethylene-dependent. As seen in Figure 3C, necrosis was as extensive in leaves of NahG and ETR plants as it was in their non-transgenic counterparts at 5 dpi (Figure 3C), indicating that the HR triggered by P19, unlike the induced antiviral state, is neither SA- nor ethylene-dependent.

sRNAs binding by P19 is necessary for P19-mediated elicitation of defense

Resolving the crystal structure of the P19-siRNA complex granted the identification of point mutations that debilitate the protein’s VSR function without impacting its stability [43]. It was notably shown that a double mutation affecting tryptophan residues 39 and 42 (W39-42R) was sufficient to abolish siRNA binding by P19 in vitro, with the resulting stable mutant allele being unable to suppress RNA silencing in planta [43]. Using the same allele, we thus tested if the capacity of P19 to sequester siRNAs was required for the elicitation of ER in N. tabacum. We generated transgenic N. tabacum cv. Xanthi lines expressing CIRV P19W39-42R under the DEX inducible promoter (DEX::P19W39-42R). Expression of P19W39-42R was quantified in two independent lines 0, 12 and 24 hours after DEX application; transgenic line Dex::P19#1, expressing WT P19 (Figure 1B), was used as a reference for functional P19 levels in these experiments. Upon DEX application onto leaves of five week old plants, quantification of both mRNA (Figure 4A) and protein (Figure 4B) levels showed that accumulation of the P19 mRNA and of P19 homo-dimers was similar in the two independent DEX::P19W39-42R tobacco lines tested and in the Dex::P19#1 reference line (Figure 4A–B).

Remarkably, P19W39-42R was neither able to induce SA accumulation, HR-like symptoms nor to promote accumulation of PR1, PR2 and PR3 compared to WT P19 (Figure 3C–D and Figure S1), suggesting that small RNA binding by P19 is necessary to trigger the onset of defense in N. tabacum. The results also show that defense elicitation can occur independently of virus infection, suggesting that binding of endogenous sRNAs by P19 is prerequisite for elicitation.

RNA silencing suppression and sRNAs binding are not sufficient, per se, to trigger HR-like lesions in N. tabacum

The above results prompted us to investigate if silencing suppression via sRNA binding was sufficient, per se, to trigger the HR-associated defense response elicited by P19 in N. tabacum. To that aim, we used Agrobacterium strains producing various VSRs unrelated to P19. HcPro from Tobacco etch virus, P15 from Peanut clump virus and P21 from Beet yellows virus are all known to bind sRNAs in vitro, with high affinity for 21 nt-long species (Figure 5A; [45]). In the same in vitro assay, P14 from Pothos latent virus was shown to bind different sizes of sRNAs ranging from 21 nt to 26 nt, while P25 from PVX was, by contrast, devoid of sRNA binding activity (Figure 5A; [45]). We found that, unlike P19, neither of the above VSRs was able to trigger the HR-like response at 5 dpi following their transient expression in leaves of N. tabacum (Figure 5B). Nonetheless, in a well-established silencing suppression assay based on transient co-expression of a silencing GFP target transgene with VSRs [66], all of these proteins were clearly able to stabilize GFP accumulation, as assessed under UV illumination (Figure 5C) and by Western analysis (Figure 5D). By contrast, GFP accumulation remained low in tissues co-infiltrated with a control Agrobacterium strain.
Co-expression of unrelated VSRs compromise the onset of HR elicited by P19, but not resistance conferred by Rx against PVX

Even though none of the above-tested VSRs triggered, on its own, a defense response in N. tabacum, the intrinsic abilities of most of these proteins to bind siRNAs predicted that their co-expression with P19 would compromise the onset of HR-like lesions observed in Agrobacterium-infiltrated tissues (Figure 1C). As shown in Figure 5E, this was indeed the case: the appearance of necrotic tissues was significantly delayed and less extensive at 96 hours in leaf patches that had received the P19-VSR co-treatments compared to leaves co-treated with P19 and GUS as a negative control (Figure 5E). Remarkably, the delayed onset of HR was not observed in co-treatments involving P19 and the P25 protein of PVX, which, unlike all the other VSRs tested, does not bind siRNAs in vitro ([45]; Figure 5E). Western analyses employing a P19 antibody also confirmed that the delayed onset of HR was unlikely to be a consequence of altered levels of P19 homodimers in the P19-VSR co-treated leaves, compared to control leaves (Figure 5F).

Given that P14, P15, P21 and Hc-Pro are all known to bind sRNA, at least in vitro [45], we assessed whether the compromised HR-like cell death phenotype observed upon concomitant expression of P19 with these VSRs resulted from a direct competition for siRNA binding, potentially decreasing the amount of P19-siRNA complexes. To address this point we transiently expressed, in N. benthamiana, a HA-tagged version of P19 (P19HA), either alone or in combination with P15 or P21 (Figure 6). As a source of siRNAs, we used a 3SS promoter-driven inverted-repeat (IR) construct, corresponding to the 5’ part (‘GF’) of the GFP sequence, which is processed into 21 nt- and 24 nt-long siRNAs.

Figure 4. Binding of small RNAs is mandatory for induction of plant immune responses by P19. (A–B) Leaves of five week old Dex::P19, Dex::P19W39-42R transgenic lines (N. tabacum cv. Xanthi) were sprayed with DEX and the kinetics of P19W39-42R accumulation at transcript (A) and protein (B) levels was analysed by qPCR and Western analysis, respectively. Actin was used as an internal control. (C) The transgenic lines described above were sprayed with DEX and appearance of an HR was assessed 5 day post-DEX application. (D) PR proteins accumulation at 0, 1 and 2 days post DEX application in Dex::P19 and Dex::P19W39-42R transgenic lines. Western analysis was conducted using anti-PR1, -PR2 and -PR3 antibodies. Coomassie or ponceau staining of the same extracts is shown to demonstrate equal protein loading. Experiments were repeated three times and showed similar results.

doi:10.1371/journal.ppat.1003435.g004
Northern analysis of the sRNA fraction of P19HA immunoprecipitates showed that, as expected, P19 specifically bound the 21 nt-long GF siRNAs. Both P15HA and P21HA displayed the same 21 nt siRNA size preference as P19 for binding. However, P21 sequestered 21 nt siRNAs significantly more efficiently than the two other VSRs, as shown by the much stronger signal detected in P21HA immunoprecipitates (Figure 6). This most likely explains the decreased GF siRNA levels observed in P19HA and P15HA immunoprecipitates (Figure 6). This most likely explains the decreased GF siRNA levels observed in P19HA and P15HA immunoprecipitates (Figure 6). This most likely explains the decreased GF siRNA levels observed in P19HA and P15HA immunoprecipitates (Figure 6).

Overall, these results show that, although necessary, the sRNA binding capacity of P19 is not sufficient for host defense elicitation in *N. tabacum*, suggesting that the onset of ER is intrinsically linked to the VSR function of P19 and not just the formation of P19-siRNA complexes *per se* (Figure 3).

To further ascertain this idea, we took advantage of the fact that Rx-mediated ER is triggered by the PVX-encoded CP protein, which does not display any intrinsic VSR activity [68]. Moreover, Rx-mediated ER can be recapitulated in transgenic *N. tabacum* upon inoculation of PVX-GFP using leaf-infiltration of *Agrobacterium*. We reasoned that, unlike in the above example where resistance was highly dependent upon the VSR function of the P19 elicitor, Rx-mediated resistance would remain unaffected by co-

**Figure 5. Effects of VSRs unrelated to P19.** (A) List of VSRs used in this study alongside their preferential sRNA binding sizes, as established *in vitro*. P25 is unable to bind sRNAs *in vitro*. Leaves of five week-old *N. tabacum* cv. Xanthi were transiently infiltrated with *Agrobacterium tumefaciens* expressing either P19, P14, P15, P21, P25 or HcPro. (B) HR response as evaluated 5 days post infiltration of the various VSRs listed in (A). (C–D) Leaves of *N. tabacum* cv. Xanthi were infiltrated with a mixture of Agrobacteria containing either P14, P15, P21 or HcPro together with a GFP transgene used as a visual and molecular reporter of the onset of RNA silencing in the co-infiltrated tissues. GFP fluorescence was visualized 4 days post-infiltration under UV light (C) and by Western analysis using an anti-GFP antibody (D). Ponceau staining of the same extracts is depicted to demonstrate equal protein loading. (E) Leaves of five week-old *N. tabacum* cv. Xanthi were infiltrated with *A. tumefaciens* strains expressing P19 in combination with either P14, P15, P21, HcPro, P25 or the GUS reporter gene. Appearance of P19-triggered HR lesions was monitored at 40 hpi (Left panel) and 96 hpi (Right panel). Experiments were repeated three times with similar results. (F) Western analysis of P19 protein levels in P19-VSR co-treatments. Proteins extracts from P19-VSRs or P19-GUS co-treated leaves were subjected to anti-P19 immunoblotting after 48 h. Ponceau staining of the membrane is shown to demonstrate equal protein loading. Experiments were repeated three times and showed similar results.

doi:10.1371/journal.ppat.1003435.g005
expression of VSRs with PVX-GFP. As shown in Figure 7, accumulation of Agrobacterium-delivered PVX-GFP was abolished in leaves of plants expressing transgenic Rx, compared to non-transgenic plants. Furthermore, this pattern remained unaffected by transient co-expression of HcPro, P21, P15 P14 VSRs, or a control GUS transgene (Figure 7).

**Discussion**

Cross-talk between RNA silencing pathways and both PTI and ETI pathways has been established experimentally in the case of bacterial pathogens [12,15]. In all cases so far, PAMP recognition activates endogenous RNA silencing pathways to target negative regulators of disease resistance, leading to potentiation of basal defense [12,15,31]. Bacterial-encoded SRs, in turn, target this basal defense by inhibiting various, and perhaps multiple, steps of host silencing pathways.

The work presented here describes how the activity of the viral suppressor P19 is sensed in specific *Nicotiana* species to induce immunity against the P19-producing virus. This immunity displays several key attributes of ETI, including the involvement of SA and ethylene, as well as the production of PR proteins. The timing of P19 homodimers accumulation correlates with the extent of cell death and PR proteins production; this is in agreement with data showed previously in which the authors used the same inducible promoter as the one we used in this study [69]. Remarkably, antiviral immunity is also accompanied by a lack of visible HR-like lesions, at least in the context of authentic tombusvirus infection, a phenomenon highly reminiscent of extreme resistance (ER) observed, for instance, during the CP-Rx interaction in PVX-infected plants. Further supporting the analogy between P19-mediated defense and the ER triggered by Rx, strong and isolated expression of their respective elicitors (i.e. P19 or CP, respectively) promotes the appearance of HR-like lesions in both cases.

Nonetheless, a marked difference between the Rx-CP and the P19 systems is the reliance of the latter upon RNA silencing suppression, a function not associated with the CP of PVX [68].

Our findings were, in fact, not completely unprecedented. Hence, the P38 capsid protein of *Turnip crinkle virus* binds AGO to inhibit its loading with sRNAs [41,42,70]. P38 was also shown to induce HR-associated defense responses in the Arabidopsis ecotype Dijon-0 and its inbred derivative Dijon-17 [71,72], a level of host specificity that strongly evokes an ETI-type of response. The elicitor of the *N* resistance gene, which confers ETI to TMV, had been also mapped to the p50 helicase subunit of the viral replicase, pl26. Remarkably, the same domain of pl26 was
recently identified as being sufficient to suppress RNA silencing in *N. benthamiana* [73]. Moreover, the helicase enzymatic activity of p50 was found dispensable for both N-mediated resistance and silencing suppression, suggesting that the VSR activity of P50 might stimulate ETI via the activation of N. Seminal work carried out more than a decade ago also provided key insights into the potential contribution of the 2b protein from *Turnip aspermy cucumovirus* (TAV2b) to the induction of ETI, possibly through its VSR activity. Indeed, when expressed from recombinant TMV, TAV2b was found to activate strong host resistance in tobacco, typical of the gene-for-gene interaction linking R proteins to their elicitors [53]. Moreover, the N-terminal region of TAV2b was found critical for both VSR activity and resistance elicitation, suggesting that the same or overlapping domains of the protein are involved [53]. Interestingly, Chen et al. [74] recently showed that TAV2b effectively binds sRNAs, highly reminiscent of the situation presented here with P19.

The seminal observation made with TAV2b led the authors to suspect that RNA silencing and its suppression on the one hand, and ETI on the other, were probably linked phenomena, at least in some cases; this view became strongly substantiated through subsequent work conducted with plant pathogenic bacteria (reviewed in [31]). The data obtained in this manuscript add further strength to this idea by showing the importance of RNA silencing suppression in the resistance mediated by P19, because immunity to TBSV was only achieved if the protein retained its capacity to suppress gene silencing, for which sRNA binding is a prerequisite. We suspect that the reported ETI-like response triggered by P19 in the absence of visible HR might also strongly contribute to its additional, albeit poorly understood, role as a host-specific determinant of systemic viral movement [51,75]. This hypothesis is particularly appealing given the involvement of SA and ethylene in the P19-elicited response in *N. tabacum*. Indeed both hormones are known to mediate, directly or indirectly, systemic, in addition to localized, defense responses.

Immune signaling pathways seem to be widely conserved across fungal, bacterial and viral interactions that lead to ETI in plants. The fact that P19-mediated resistance was compromised by many unrelated VSRs, unlike resistance activated by Rx argues, therefore, against an interference at the level of disease resistance signaling. Moreover, the PVX coat protein (elicitor of Rx) does not possess VSR function [68]. The fact that the integrity of the P19 binding domain is required for defense elicitation, together with the failure of PVX P25, among the VSR tested here, to alter the P19-mediated HR response, suggests that sRNA binding, required for VSR function, is a key component for defense activation in *N. tabacum*. It is, however unlikely to be sufficient, because none of the other VSRs tested was able to recapitulate, on its own, the defense phenotype induced by P19 when transiently expressed, despite that many of them bind sRNA in *vivo* and probably in *vivo*. Additionally, P15 could suppress the P19-mediated HR even though it did not outcompete P19 for sRNA binding in the *N. benthamiana* transient expression assay. The simplest interpretation of these results, therefore, is that P19 dimers complexed with sRNAs initiate a signal that is specifically sensed in *N. tabacum* to trigger extreme resistance against TBSV or that a conserved motif or structure important for sRNA binding by P19 is sensed in the plant. A non-mutually exclusive possibility holds that sensing occurs downstream, as a consequence of specific P19-sRNA association in a manner suppressed by the action of VSRs such as P15, which may share downstream silencing targets with P19 including AGOs. Interestingly, HR-like lesions and *PR* proteins accumulation could be triggered by P19 in the absence of a viral infection, suggesting that endogenous sRNAs, including sRNAs and miRNAs, which are effectively bound by P19 together with viral-derived siRNAs during infection [43,49,67,76], form one component of the trigger. Hence, a recent study in transgenic Arabidopsis shows that binding of endogenous miRNAs by VSRs is much less widespread than was originally anticipated. In fact, P19 was, among many VSRs tested (including several used in the present study), the only protein to prevent loading of miRNAs into AGO1. By contrast, all of the VSRs tested could effectively prevent loading of exogenous siRNAs into AGO1 [42]. This peculiarity may contribute to explain the specific ability of P19 to trigger HR-like lesions and ER in *N. tabacum*. It is also possible that the binding of P19 to si/miRNAs promotes a specific change in the integrity or conformation of silencing effector proteins, including AGOs, and that these changes are sensed in a host-specific manner.

miRNAs have roles in plant basal and race-specific resistance against bacterial pathogens [15,16,77]. Furthermore, some plant miRNAs appear to have evolved to control R gene expression presumably to prevent the known fitness cost of their constitutive expression in the absence of pathogens [78–80]. For example, nta-miR6019 (22-nt) and nta-miR6020 (21-nt) guide the cleavage of the TIR-NB-LRR _N_ transcript from tobacco, which confers resistance to *Tobacco mosaic virus* [79]. Likewise, Sl-miR1482 attenuates expression of a large family of NBS-LRR genes from tomato and its accumulation is decreased in plants infected with *Turnip crinkle virus*, *Cucumber mosaic virus*, *Tobacco rattle virus* and *Pst DC3000* [80]. Therefore, given the above context, miRNA sequestration by P19 might generally enhance host immune responses induced by virulent and avirulent pathogens. Interestingly, however, miR168, which targets the antiviral silencing effector AGO1, is specifically not sequestered and, in fact, induced by P19, suggesting that, in this case, miRNA binding by P19 favours viral infection without activating immune responses [81].

We have shown here, with the P19- *N. tabacum* model, that the general scheme of silencing induction and suppression by plant viruses can be readily accommodated within the classical frame of ETI/PTI. In particular, our study sheds light on an additional layer of defense, whereby hosts can sense and respond to the damages caused by VSRs to the cellular silencing machinery. The existence of this additional layer is also consistent with the fast evolving and highly diverse nature of VSRs. Indeed, potent host counter-counter-defense measures probably impose strong selective pressure on pathogens to accelerate or refine the modeling of their virulence factors, thereby contributing further to the never-ending arms race opposing parasites to their hosts. A future challenge will be to assess the extent to which the phenomenon described here is shared not only among plant-virus, but also plant-bacteria, plant-fungal and plant-oomycetes interactions, and how elucidation of its biochemical and genetic underpinnings might improve our understanding of PTI and ETI at large. Finally, and most importantly, a strong -albeit still speculative- implication of our results is the existence of dedicated host-encoded R proteins that should monitor the status of key RNA silencing components in plants, and perhaps other organisms. Identifying these elusive silencing-associated R proteins and their guardees would certainly constitute a major breakthrough in the field.

**Materials and Methods**

**Plant conditions and transgenic lines**

Wild type and transgenic plants were grown under conditions of 8 h darkness at 19°C, 16 h light at 22°C, with 70% relative humidity. Independent tobacco (*N. tabacum*) transgenic lines

carrying the wild type P19 and its mutant P19W39-42R under Dex inducible promoter [59] were generated using the Agrobacterium tumefaciens leaf disc transformation method [82]. The disarmed pTA7001-Dex-P19, pTA7001-Dex-P19W39-42R were used for transformation. The transgenic plants generated were named Dex::P19 and Dex::P19W39-42R.

Transgenic expression

A. tumefaciens strains containing the constructs P19 [35], P25 [68], P15 [83], HcPro [67], P14 and P21 [45] were grown overnight at 28°C in Lauria Bertani (LB) broth supplemented with 50 μg/ml kanamycine, 10 μg/ml rifampicin and 25 μg/ml gentamycine. Bacterial cultures were then pelleted at 4 500 g for 15 min and the supernatant was discarded. Pellets were resuspended in 10 mM MgCl2 supplemented with 200 μM acetoxyxime and brought to an OD 0.5. These bacterial suspensions were infiltrated in the plant leaves using a syringe. Co-agroinfiltration of mGFP and VSRs were done at 0.5 OD.

Protein extraction and gel blot analysis

For transgenic plants, a solution of 25 μg/ml Dexametason supplemented with 0.1% v/v Silwet L-77 was sprayed onto leaves of 5 week-old transgenic plants. Samples were collected 12, 24 and 48 hours after DEX application, immediately frozen in liquid nitrogen, and kept at −80°C before extraction.

Total proteins were extracted from 100 to 200 mg of homogeny of frozen leaf 200 μl of extraction buffer [25 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 5 mM dithiothreitol (DTT)] and a protease inhibitor cocktail (Sigma). The crude extract was centrifuged at 12000 g for 15 min. The supernatant was kept and total proteins were quantified by Bradford assay (Bio-Rad Laboratories, Ontario). Samples were diluted in Laemmli buffer and boiled for 5 minutes before separation on 12% SDS-PAGE. 50 μg of proteins of each sample was used for Western analysis. Protein samples were subjected to gel blot analysis using a rabbit polyclonal PR1, PR2 or PR3 antibodies, at a dilution of 1 : 8000 [84]. For detection of the GFP, a rabbit polyclonal IgG antibody was used at 1/300. Immunoaa detection was conducted with chemiluminescent rabbit IgG was used as secondary antibody at 1 : 14500 (Sigma Aldrich). Immunodetection was conducted with chemiluminescent substrate (Bio-Rad, immuno-star kit) followed by X-ray film exposure.

Quantitative PCR

Total RNA was extracted from tobacco tissues using the RNeasy Plant Mini Kit (Qiagen Science, Maryland, USA) according to the manufacturer’s instructions. 2 μg of each RNA samples were reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen). Samples were diluted 1/5 in DEPC water and qPCR was performed using POWER SYBR Green (Applied Biosystems, Warrington, UK) according to the manufacturer’s instruction. Primers used were: qPCR NTAc1F 5'-CTGTATACACTACGTTAGGACACTTG-3', qPCR NTAc1R 5'-GAGGACATATACAGCAGGA-3', qPCR P19R 5'-GGATCAAGGACGGTGC-3', qPCR P19F 5'-TTTGGTTTAAAGGAAGCTG-3', qPCR NTAC1R F 5'-GATCGAAGCTCTGTGCA-3', qPCR1.

Virus infections

A. tumefaciens strains containing the constructs 35S::TBSV-GFP or 35S::TBSVΔP19-GFP (Kindly provided by Herman B. Scholthof [26]) were grown overnight at 28°C in Lauria Bertani (LB) broth supplemented with 50 μg/ml kanamycine, 10 μg/ml rifampicin and 25 μg/ml gentamycine. Bacterial cultures were then pelleted at 4 500× g for 15 min and the supernatant was discarded. Pellets were resuspended in 10 mM MgCl2 brought to 0.5 OD and supplemented with 200 μM acetoxyxime. Bacterial suspensions were then incubated at room temperature for 1–3 hours before being infiltrated into young leaves of 5 week-old Nicotiana tabacum plants, using a syringe. Inoculated plants were grown under conditions of 8 h darkness at 18°C, 16 h light at 20°C with 70% relative humidity. Viral infection was monitored over time under U.V. illumination and samples were collected at 6 dpi, frozen in liquid nitrogen and kept at −80°C before extraction of protein or RNA. A. tumefaciens strain containing the construct 35S::PVX-GFP [53] was used for PVX assays. Infections were conducted as described above, except that the final OD used was 0.25. VSR were co-agroinfiltrated at final OD of 0.25.

Northern analysis

Total RNA was extracted using TRI reagent (Sigma), precipitated with isopropanol and the RNA pellet was resuspended in 50% denized Formamide. Analysis was performed as described [66]. The signal was detected using X-ray films.

Trypan blue staining

Samples were boiled for 5 minutes in the staining solution [10 ml of lactic acid, 10 g of phenol, 10 ml of glyceral, 10 ml of water, 10 mg of trypan blue, mixed 1:1 with ethanol]. Samples were then destained using chloral hydrate as previously described [86,87].

Immunoprecipitation experiments

The cassettes for transient expression of GFFG dsRNA and silencing suppressors have been described previously [66,67]. Agrobacterium-mediated transient expression in N. benthamiana leaves was as described previously [88].

For immunoprecipitation experiments, 400 μg of frozen tissue harvested 5 days post-infiltration was ground in liquid nitrogen and homogenized in 3 ml of extraction buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glyceral, 0.1% NP-40 and complete protease inhibitor cocktail (Roche) for 30 min at 4°C. Cell debris was removed by centrifugation at 12000 g at 4°C for 30 min. Extracts were pre-cleared by incubation with Protein A-agarose (Roche) at 4°C for 1 h. Pre-cleared extracts were then incubated with anti-HA polyclonal antibody (Sigma) and protein A-agarose overnight at 4°C. Immunoprecipitates were washed three times (15 min each) in extraction buffer. Aliquots of the inputs and immunoprecipitates were collected for protein blot analysis. For RNA analysis, immune complex were subjected to Tri-Reagent extraction (Sigma).

Salicylic acid quantification

An amount of one to one v/v of cold 80% MeOH was added to finely ground plant tissue (300–500 mg) for extraction of phenolic compounds. Samples were vortexed then shaken overnight at 4°C. The following morning, the samples were vortexed and centrifuged 16,000 g for 10 min. The supernatant was transferred to a new Eppendorf tube, filtered through a 0.22-μm syringe filter and centrifuged 16,000 g for 10 min. The supernatant was transferred to a new Eppendorf tube, filtered through a 0.22-μm syringe filter and subjected to lactic acid, 10 g of phenol, 10 ml of glyceral, 10 ml of water, 10 mg of trypan blue, mixed 1:1 with ethanol]. Samples were then destained using chloral hydrate as previously described [86,87].

Suppressor of RNA Silencing and Extreme Resistance
detected with a Waters 996 diode array scanning 200 nm–400 nm, followed, in tandem, by a Waters 2475 Fluorescence detector, with an excitation wavelength of 290 nm emission and a scan of 300–500 nm. The maximum expected emission for free salicylic acid using this excitation wavelength was at 390–400 nm. The HPLC system was controlled and data analysed with the Empower2 software. Standard free salicylic acid (Sigma 84210) standards were prepared at 100 ng/ml, 250 ng/ml, 500 ng/ml, 1000 ng/ml and injected under the same conditions. The solvents were acidified water (solvent A: 0.1% Phosphoric acid in nanopure water) and acetonitrile HPLC grade (solvent B) with an elution flow rate of 1 mL/min. The gradient used was as follows: time (min)/%A/%B: 0/100/0, 5/95/5, 10/95/5, 14/90/10, 20/80/20, 23/80/20, 30/65/35, 35/65/35, 43/50/50, 48/25/75, 55/0/100 and 60/0/100. The injected volume was 50 μL for each sample. Three biological replicates for each treatment/time point were extracted and injected independently into the HPLC. Linear regressions were generated between compound concentration (independent variable) and peak areas (dependent variable). The equations obtained were used to calculate the concentration of each phenolic compound in the samples were harvested at 0 and 24 hours post treatment (hpt) for SA treatments. Dex::P19 and Dex::P19W39-42 plants were sprayed with Dex and samples were extracted and injected independently into the HPLC. The injected volume was 50 μL for each sample. Three biological replicates were used to confirm the results. We also thank Dr Kauffmann for providing antibodies for PR proteins, Dr Herman Scholthoff for TBSV constructs and Dr Moffett for PVX constructs.

Supporting Information

Figure S2 TBSV does not induce microscopic HR in N. tabacum. (A–B) Five week-old N. tabacum cv. Xanthi plants were transiently infiltrated with a solution of A. tumefaciens expressing either GUS, TBSV-GFP or P50 and 3 and 5 dpi leaves were stained with trypan blue to see macroscopic (A) and microscopic HR (B). Experiments were repeated at least three times and gave similar results.

References

1. Jones JD, Dangl JL (2006) The plant immune system. Nature 444: 323–329.
2. Zipfel C (2009) Early molecular events in PAMP-triggered immunity. Curr Opin Plant Biol 12: 414–420.
3. Dodds PN, Rathjen JP (2010) Plant immunity: towards an integrated view of plant-pathogen interactions. Nature Rev Genet 11: 539–549.
4. van Loon LC, Rep M, Pieterse CMJ (2006) Significance of Inducible Defense-Related Proteins in Infected Plants. Annu Rev Phytopathol 44: 135–162.
5. Bent AF, Mackey D (2007) Effectors, effectors, and R genes: The new paradigm and a lifetime supply of questions. Annu Rev Phytopathol 45: 399–436.
6. Fung B, Heitz T, Legendre M (1998) Antimicrobial proteins in induced plant defence. Curr Opin Immunol 10: 16–22.
7. Oh H-S, Park DH, Gallner A (2010) Components of the Pseudomonas syringae Type III Secretion System Can Suppress and May Elicit Plant Immune Defense. Mol Plant Microbe Interact 23: 727–739.
8. Bendahmane A, Kanyuka K, Baulcombe DC (1999) The Rx Gene from Potato Requires its Capacity to Bind sRNAs. Cell 97: 781–792.
9. NAVarro L, Dounoy P, Jay F, Arnold B, Dharmasiri N, et al. (2006) A Plant miRNA Contributes to Antibacterial Resistance by Regressing Auxin Signaling. Science 312: 436–439.
10. Katiyar-Agarwal S, Jin H (2010) Role of Small RNAs in Host-Microbe Interactions. Annu Rev Phytopathol 48: 225–246.
11. Zhang Y, Yang W-K, Gao L (2011) Evolution of MicroRNA Genes in Oryza sativa and Arabidopsis thaliana: An Update of the Inverted Duplication Model. PLoS ONE 6: e20873.
12. Hamilton AJ, Baulcombe DC (1999) A Species of Small Antisense RNA in Posttranscriptional Gene Silencing in Plants. Science 286: 950–952.
13. Elbashir SM, Lendeckel W, Tuschl T (2001) RNA interference is mediated by 21- and 22-nucleotides RNAs. Genes Dev 15: 188–200.
14. Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409: 363–366.
15. Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclelease mediates post-transcriptional gene silencing in Drosophila cells. Nature 409: 293–296.
16. DeJonge J, Balsalobre-Jao B, Kasschau K, Carrington J, et al. (2006) Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. Science 313: 68–71.
17. Qu F, Ye X, Morris JT (2006) Arabidopsis DRA4, AGO1, AGO7, and RDR6 participate in a DCL1-initiated antiviral RNA silencing pathway negatively regulated by DCL1. Proc Natl Acad Sci USA 103: 14732–14737.
18. Harvey JJ, Lewsey MG, Patel K, Westwood J, Heimstadt S, et al. (2011) An antiviral defense role of AGO2 in plants. PLoS One 6: e16439.
19. Jaubert M, Bhattacharjee S, Mello AFS, Perry KL, Moffett P (2011) ARGONAUTE2 Mediates RNA-Silencing Antiviral Defences against Potato virus X in Arabidopsis. Plant Physiol 156: 1536–1540.
20. Scholthoff HB, Alvarado VV, Vega-Arreguin JC, Compierik J, Odokoneryo D, et al. (2011) Identification of an ARGONAUTE for Antiviral RNA Silencing in Nicotiana benthamiana. Plant Physiol 156: 1536–1540.
21. Wang XR, Jovel J, Udoornporn P, Wang Y, Wu Q, et al. (2011) The 21-nucleotide, but not 22-nucleotide, viral secondary small interfering RNAs direct
potent antitumor defense by two cooperative argonautas in Arabidopsis thaliana. Plant Cell 23: 1625–1638.

28. Garcia D, Garcia S, Portier D, Marchais A, Renous Jean P, et al. (2012) Agro Hook and RNA Helicase Mootain Underpin Dual Roles for SDE3 in Antiviral Defense and Silencing of Nonconverged Intergenic Regions. Mol Cell 48: 109–20.

29. Brehm E, Lohuis D, van Poppel PMJ, Geerts-Dimitriadis C, Goldbach R, et al. (2006) Multiple virus resistance at a high frequency using a single transgene against tomato bushy stunt virus. J Virol 80: 3937–3947.

30. Voinnet O (2009) Origin, biogenesis, and activity of plant microRNAs. Cell 136: 669–687.

31. Ruiz-Ferrer V, Voinnet O (2009) Roles of Plant Small RNAs in Biotic Stress Responses. annu rev plant biol 60: 495–516.

32. Anandalakshmi R, Pruss GJ, Ge X, Marathe R, Mallory AC, et al. (1998) A viral suppressor of gene silencing in plants. Proc Natl Acad Sci USA 95: 13079–13084.

33. Brogini G, Voinnet O, Li W-X, Ji L-H, Ding S-W, et al. (1998) Viral suppression of transgene gene silencing in Nicotiana benthamiana. EMBO J 17: 6739–6746.

34. Baulcombe DC, Molnar A (2004) Crystal structure of p19 - a universal suppressor of RNA silencing. Trends Biochem Sci 29: 279–281.

35. Schott G, Mari-Ordonez A, Himber C, Alioua A, Voinnet O, et al. (2012) The Tombusvirus-encoded P19: from irrelevance to Relevance of a Stable Biochemical Interaction between the Tombusvirus-encoded P19 and Short Interfering RNAs. J Virol 80: 3000–3008.

36. Navarro L, Jay F, Nomura K, He SY, Voinnet O (2008) Suppression of the RNA silencing encoded by peanut clump virus. Plant J 29: 555–567.

37. Choi CW, Qu F, Ren T, Ye X, Morris TJ (2004) RNA silencing-suppressor function of Turnip crinkle virus coat protein cannot be attributed to its interaction with the Arabidopsis protein TIP1. J Gen Virol 85: 3415–3420.

38. Garcia D, Garcia S, Portier D, Marchais A, Renous Jean P, et al. (2012) Agro Hook and RNA Helicase Mootain Underpin Dual Roles for SDE3 in Antiviral Defense and Silencing of Nonconverged Intergenic Regions. Mol Cell 48: 109–20.

39. Brehm E, Lohuis D, van Poppel PMJ, Geerts-Dimitriadis C, Goldbach R, et al. (2006) Multiple virus resistance at a high frequency using a single transgene against tomato bushy stunt virus. J Virol 80: 3937–3947.

40. Voinnet O (2009) Origin, biogenesis, and activity of plant microRNAs. Cell 136: 669–687.

41. Ruiz-Ferrer V, Voinnet O (2009) Roles of Plant Small RNAs in Biotic Stress Responses. annu rev plant biol 60: 495–516.

42. Anandalakshmi R, Pruss GJ, Ge X, Marathe R, Mallory AC, et al. (1998) A viral suppressor of gene silencing in plants. Proc Natl Acad Sci USA 95: 13079–13084.

43. Brogini G, Voinnet O, Li W-X, Ji L-H, Ding S-W, et al. (1998) Viral suppression of transgene gene silencing in Nicotiana benthamiana. EMBO J 17: 6739–6746.

44. Baulcombe DC, Molnar A (2004) Crystal structure of p19 - a universal suppressor of RNA silencing. Trends Biochem Sci 29: 279–281.

45. Voinnet O, Pinto YM, Baulcombe DC (1999) Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses. Proc Natl Acad Sci USA 96: 14147–14152.

46. Navarro L, Jay F, Nomura K, He SY, Voinnet O (2008) Suppression of the MicroRNA Pathway by Bacterial Effector Proteins. Science 321: 964–967.

47. Qiao Y, Liu L, Xiong Q, Flores C, Wong J, et al. (2013) Osmoyer pathogens encode RNA silencing suppressors. Nat Genet 45: 330–333.

48. Baumberger N, Tsai CH, Lie M, Havecker E, Baulcombe DC (2007) The Polerovirus silencing suppressor P0 targets ARGONAUTE proteins for degradation. Curr Biol 17: 1699–1704.

49. Bortolamiel D, Pazhouhandeh M, Marrocco K, Genschik P, Ziegler-Graff V (2007) The Poliovirus F box protein P0 targets ARGONAUTE1 to suppress RNA silencing. EMBO J 26: 1615–1621.

50. Cserbo T, Lozsa R, Hutvagner G, Burgyan J (2010) Poliovirus protein P0 prevents the assembly of small RNA-containing RISC complexes and leads to degradation of ARGONAUTE1. Plant Cell 22: 463–472.

51. Aszédov J, Garcia D, Portier D, Ohnesorge S, Yu A, et al. (2010) Argonauta quenching and global changes in Dicer hostonastasis caused by a pathogen-encoded GW repeat protein. Genes Dev 24: 904–915.

52. Schott G, Mari-Ordóñez A, Himber C, Aloua A, Voinnet O, et al. (2012) Differential effects of viral silencing suppressors on siRNA and miRNA loading suggest two distinct cellular pools of ARGONAUTE1. EMBO J 31: 2553–2565.

53. Vargason J, Szitya A, Burgyan J, Tanaka Hall T (2003) Size selective recognition of siRNA by an RNA silencing suppressor. Cell 113: 799–811.

54. Lakatos L, Cserbo T, Pantaleo V, Chapman E, Carrington J, et al. (2006) Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. EMBO J 25: 2768–2780.

55. Merai Z, Kereven Z, Kertesz S, Magna M, Lakatos L, et al. (2006) Double-stranded siRNA synthesis may be a general plant viral strategy to suppress RNA silencing. J Virol 80: 5747–5756.

56. Cserbo T, Boev D, Dalmay T, Burgyan J (2007) The P22 subunit of Tobacco Mosaic Virus replicase is a potent silencing suppressor and compromises both small interfering RNA- and miRNA-mediated pathways. J Virol 81: 11768–11780.

57. Hemmes H, Lakatos L, Goldbach R, Burgyan J, Prins M (2007) The NS3 protein of Rice hoja blanca tungro virus suppresses RNA silencing in plant and insect hosts by efficiently binding both siRNAs and miRNAs. RNA 13: 1079–1089.

58. Ye K, Malinina L, Patel DJ (2003) Recognition of small interfering RNA by a viral suppressor of RNA silencing. Nature 126: 874–876.

59. Lakatos L, Szitya A, SiIhavy D, Burgyan J (2006) Molecular mechanism of RNA silencing suppression mediated by P19 protein of tombusviruses. EMBO J 23: 873–881.

60. Omary R, Sparks K, Smitl M, Zwindic J, Schlotherb HU (2006) Biological Relevance of a Stable Biochemical Interaction between the Tombusvirus-encoded P19 and Short Interfering RNAs. J Virol 80: 3900–3908.

61. Schlotherb HU (2006) The Tombusvirus-encoded P19: from irrelevance to elegance. Nat Rev Micro 4: 405–411.

62. Jay F, Wang Y, Yu A, Tacoratali, Pelleret S, et al. (2011) Misregulation of a UNX1 RESPONSE FACTOR gene underlies the developmental abnormalities caused by three distinct viral silencing suppressors in Arabidopsis. PLoS Pathog 7: e1002035.

63. Li W-H, Lacy AP, Guo HS, Li W-X, Ji L-H, et al. (1999) Strong host resistance targeted against a viral suppressor of the plant gene silencing defense mechanism. EMBO J 18: 2663–2669.

64. Schlothohs H, Schlothofh K, Jackson A (1995) Identification of tomato bushy stunt virus host-specific symptom determinants by expression of individual genes from tobacco mosaic virus replicase. Plant Cell 7: 1517–1528.

65. Cha M (2000) Genetic dissection of tomato bushy stunt virus p19-protein mediated host-dependent symptom induction and systemic invasion. Virology 266: 79–87.
84. Cordelier S, de Ruffray P, Fring B, Kauffmann S (2003) Biological and molecular comparison between localized and systemic acquired resistance induced in tobacco by a Phytophthora megasperma glycoprotein elicitor. Plant Mol Biol 51: 109–116-118.
85. Peart JR, Cook G, Feys BJ, Baulcombe DC (2002) An EDS1 orthologue is required for N-mediated resistance against tobacco mosaic virus. Plant J 29: 569–579.
86. Bouarab K, Melton R, Peart J, Baulcombe D, Osbourn A (2002) A saponin-detoxifying enzyme mediates suppression of plant defences. Nature 418: 889–892.
87. El Oirdi M, Bouarab K (2007) Plant signalling components EDS1 and SGT1 enhance disease caused by the necrotrophic pathogen Botrytis cinerea. New Phytol 173: 131–139.
88. Hamilton A, Voinnet O, Chappell L, Baulcombe DC (2002) Two classes of short interfering RNA in RNA silencing. EMBO J 21: 4671–4679.