Minireview

Bacterial pathogens encode suppressors of RNA-mediated silencing

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Abstract

Plant pathogenic bacteria encounter host defenses mediated by a variety of small RNAs. Bacterial suppressors of silencing that inhibit multiple steps of plant microRNA biogenesis and function have recently been identified.

Resistance of plants to the pathogenic bacteria that infect them involves elaborate defense and counter-defense systems. The initial plant defense reaction, known as PAMP-triggered immunity (PTI), follows from the recognition of one of many 'pathogen-associated molecular patterns' (PAMPs), such as components of the bacterial flagellum or the bacterial translational machinery (Figure 1) [1]. To counteract PTI, bacteria inject dozens of 'effector' proteins into the plant cell via a specialized secretion system. These effector proteins alter the plant cell's transcriptome and proteome to reprogram the cell to become susceptible to the pathogen. If the effector molecules are recognized by the host cell, they trigger an additional layer of defense known as effector-triggered immunity (ETI; see Figure 1) [1]. In the past few years, silencing of plant gene expression via host-encoded small RNAs has been implicated in both PTI and ETI [2,3]. Work from the laboratory of Olivier Voinnet published recently in *Science* (Navarro *et al*. [4]) sheds further light on this struggle between pathogen and host by identifying a mechanism by which the bacterial pathogen can suppress the RNA-mediated silencing of endogenous gene expression that helps set up the state of PTI.

The first evidence that small RNAs were involved in plant defense and bacterial counter-defense came from the analysis of changes in the plant transcriptome induced by one of the bacterial effector molecules - AvrRpt2. Recognition of this effector protein by the host cell induces convergent transcription at a locus in the plant genome such that the overlapping and complementary transcripts can anneal to generate a double-stranded (ds) RNA [2]. This dsRNA is then processed into a type of small RNA known as natural antisense small interfering RNA (nat-siRNA), which is typically 21 to 24 nucleotides long. The small RNAs induced by AvrRpt2 silence protein-coding genes in the plant genome and their effect is to enhance ETI. Similarly, recognition of a peptide from bacterial flagellin has been shown to induce accumulation of another small RNA, the microRNA (miRNA) miR393, which represses the production of proteins involved in auxin signaling in the plant cell, in this case enhancing the first line of defense - PTI [3].

The recent work from Voinnet's laboratory [4] provides further support for the involvement of RNA-mediated silencing in the PTI defense against bacteria. They show that loss of miRNA pathways in *Arabidopsis thaliana* results in loss of PTI against an *Arabidopsis* pathogen, *Pseudomonas syringae*, and loss of the similar 'innate' resistance to plant pathogenic bacteria that do not normally infect *Arabidopsis* (non-host resistance). They go on to uncover distinct effector proteins - known as silencing suppressors - from *P. syringae* that inhibit this RNA-mediated component of PTI. A final part of their analysis may have relevance to crop plants in the field, because they show how there can be synergism between viral and bacterial pathogens due to the ability of their silencing suppressors to target different stages in the plant's gene-silencing pathways.

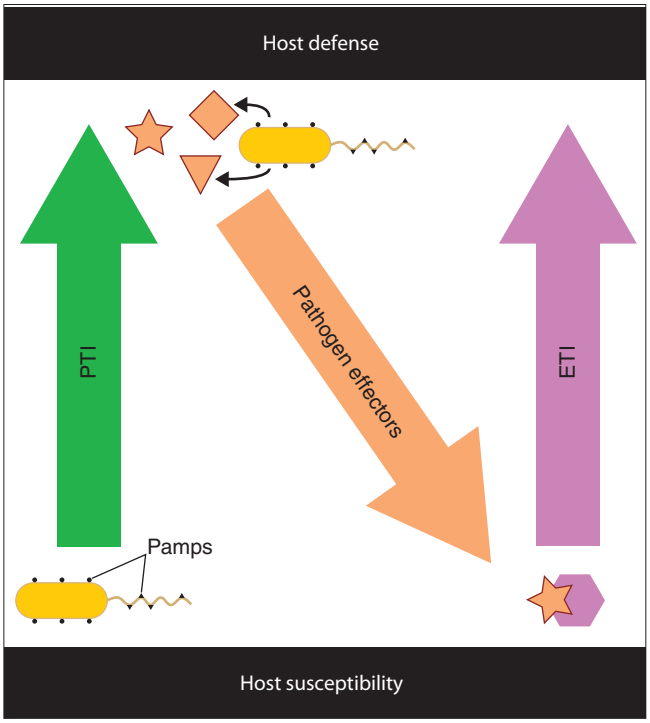

Figure 1
Multiple lines of plant defenses against bacterial pathogens. Recognition of pathogen-associated molecular patterns (PAMPs) by the plant cell induces PAMP-triggered immunity (PTI; green arrow), which involves the coordinated expression and repression of plant-cell genes to combat pathogen growth. Bacterial pathogens such as *Pseudomonas syringae* inject effector proteins (orange shapes) into the host cell to reprogram the proteome and transcriptome for susceptibility (orange arrow). Recognition of effector proteins by host proteins (purple hexagon) induces a second line of defense, effector-triggered immunity (ETI; purple arrow).

MicroRNAs are required for first-line resistance

To investigate the involvement of small RNAs in disease resistance, Voinnet and colleagues analyzed the *A. thaliana* mutants *dcl1* and *hen1*, which are defective in components of pathways that produce small RNAs. The wild-type gene *DCL1* encodes the enzyme Dicer, which is responsible for precursor miRNA processing, and *HEN1* encodes an enzyme that methylates the 2' hydroxyls of siRNAs and miRNAs to protect them from degradation [5]. Strong mutant alleles at either locus cause an almost total absence of miRNA accumulation, including that of the PAMP-responsive miR393. In addition, the *hen1* mutant displays a dramatic loss of endogenous siRNAs, including nat-siRNAs. These mutants were infected with *P. syringae* pv. *tomato* strain DC3000 (*Pto* DC3000) in which the effector molecule secretion system had been eliminated. This mutant strain is recognized by the plant, but cannot inject effector proteins into the host cell and therefore grows very weakly on a wild-type plant. On *dcl1* or *hen1* mutants, however, bacterial growth was significantly increased, indicating that these mutants are defective in PAMP perception or signaling. Enhanced bacterial growth on the miRNA-defective mutant was not surprising, as these plants cannot produce the PAMP-responsive miR393, which is known to aid resistance to *Pto* DC3000. It is notable, however, that *hen1* and *dcl1* allow a similar level of pathogen growth, whereas none of the other small RNA mutants tested, including the *dcl2 dcl3 dcl4* triple mutant, which produces miRNAs but no other small RNAs, allows pathogen growth. This difference indicates that miRNAs and not siRNAs are involved in PTI.

Strikingly, when plant pathogenic bacteria that do not normally infect *Arabidopsis* are inoculated into *dcl1* or *hen1* plants, they also exhibit enhanced growth. On a wild-type host these bacteria trigger PTI, but are unable to overcome it as they do not produce the appropriate effector proteins [1]. On miRNA-mutant hosts PTI is compromised and so the pathogens are successful. It will be interesting to discover whether the effect seen in the miRNA mutants is due to the loss of many miRNAs or only of miR393, the only miRNA known until now to affect disease resistance. As we discuss later, Voinnet and colleagues [4] give evidence that multiple miRNAs are affected by pathogen infection, but the significance of these changes in relation to disease is unknown.

The growth of both the secretion-defective *Pto* DC3000 and the 'non-host' pathogens on *Arabidopsis dcl1* and *hen1* mutants is not as robust as the growth of *Pto* DC3000 with an intact secretion system. This perhaps indicates that miRNAs play a role in host susceptibility as well as in resistance and that only their cumulative effect can be observed in mutants. The lack of robust pathogen growth in miRNA mutants may also be due to the pleiotropic nature of these mutations. Strong alleles of *dcl1* and *hen1* have morphological phenotypes that may affect pathogen growth in a way that is unrelated to the defense pathways discussed here. Comparing the growth of virulent *Pto* DC3000 on these mutants would help answer this question.

Bacterial effector proteins suppress steps in the microRNA pathway

The short life cycles of bacteria and the extreme selective pressure of a pathogenic lifestyle make it likely that bacteria have evolved means to evade or combat miRNA-mediated resistance. Just as plant viruses are known to have evolved multiple suppressors of silencing [6-9], so bacteria may have evolved bacterial suppressors of silencing. With this hypothesis in mind, Voinnet and colleagues [4] searched through *Pto* DC3000 effector molecules for those that affect miRNA biogenesis, maturation and function (Figure 2).

They found that the effector protein AvrPtoB, when expressed transiently in *Arabidopsis* leaves, causes a decrease in the accumulation of the miR393 precursors primary miR393a (pri-miR393a) and pri-miR393b. This effect is likely to be at the transcriptional level, as a miR393

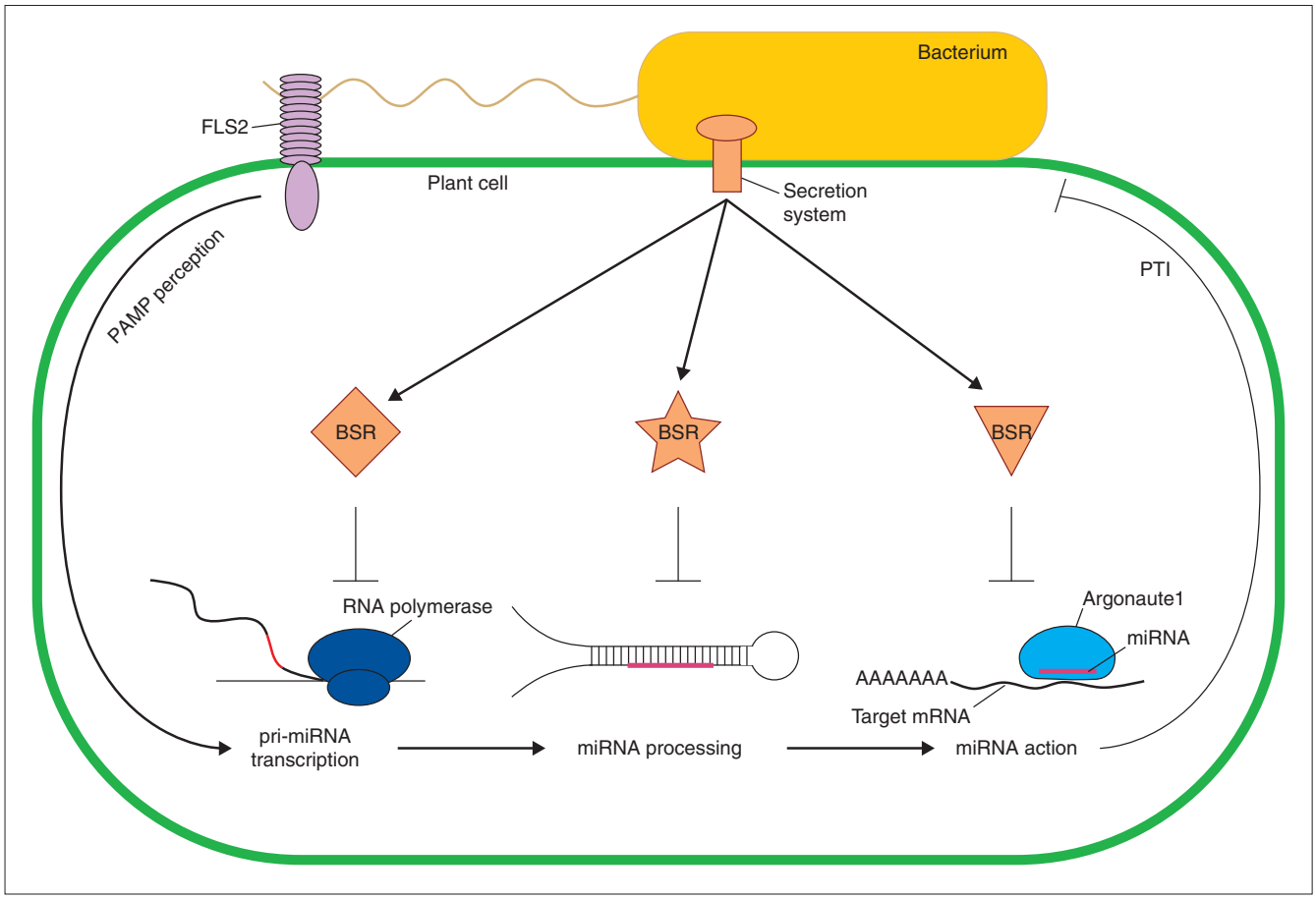

Figure 2
Bacterial suppressors of silencing interfere with miRNA-mediated host defense. Bacterial infection is initially perceived by the plant cell through PAMPs, as in the recognition of flagellin by the plant receptor FLS2 illustrated here. This triggers a signaling cascade that activates transcription of primary-microRNAs (pri-miRNAs) from the plant genome, which are then processed into mature miRNAs (red line). miRNA is bound by the protein Argonaute1 (light blue) and the miRNA-Argonaute1 complex silences specific mRNAs, enhancing resistance to the pathogen (PTI). The pathogen injects numerous effector proteins (orange shapes) into the host cell in an attempt to reprogram the cell to become susceptible to the pathogen. Voinnet and colleagues [4] recently identified some of these effector proteins as bacterial suppressors of silencing (BSRs), which inhibit the miRNA pathway at multiple stages to inhibit host defense.

promoter-green fluorescent protein reporter construct is transcribed at a reduced rate in the presence of AvrPtoB both before and after induction by a PAMP. This observation is consistent with a known role for AvrPtoB in suppression of other PAMP-responsive genes. In addition to miR393, several other miRNAs are reported to increase upon infection with secretion-defective *Pto* DC3000 [10]. Of these, miR396 at least appears to be transcriptionally repressed by AvrPtoB. However, it is unlikely that AvrPtoB functions as a general repressor of miRNA transcription, as Voinnet and colleagues [4] show that at least some pri-miRNAs are unaffected by AvrPtoB. Whether this silencing suppressor is capable of repressing non-PAMP-responsive pri-miRNAs is unknown.

AvrPto, an effector molecule unrelated to AvrPtoB, also functions as a suppressor of silencing. When Voinnet and colleagues [4] transiently expressed AvrPto in wild-type *Arabidopsis* they found a decrease in the accumulation of miR393, miR171 and miR173. Likewise, in stable transgenic *Arabidopsis* lines that carry an inducible AvrPto, the authors found that mature miRNA levels decreased upon induction without alteration in the transcription rate of pri-miRNAs. For miR393 at least, accumulation of a partially processed RNA is detected, supporting the conclusion that AvrPto interferes with miRNA processing. Voinnet and colleagues [4] also identify two other bacterial effector proteins, HopH1 and HopN1, that affect mature miRNA accumulation without reducing pri-miRNA levels, although they cannot rule out the possibility that these proteins affect miRNA stability rather than processing.

It will be particularly interesting to know whether these proteins suppress the processing of all small RNAs, all

miRNAs, or only a subset of miRNAs. Voinnet and colleagues [4] find that HopH1 and HopN1 reduce accumulation of the *trans*-acting siRNA tas255, hinting that they may affect a conserved process in small RNA processing; however, production of this siRNA requires the action of a miRNA, so the effect of these silencing suppressors may be specific to a subset of miRNAs. Were these proteins to broadly inhibit small RNA maturation they would be a powerful tool for research on small RNAs.

The final step at which bacterial effector proteins might suppress miRNA-mediated defense is by inhibiting the action of miRNAs. Bound into Argonaute1, miRNAs can silence gene expression by cleaving mRNAs or by inhibiting their translation [5,11]. Voinnet and colleagues [4] find that the effector protein HopT-1 appears to interfere with both functions when stably expressed in *Arabidopsis*. The mRNA levels of several miRNA targets increase when HopT-1 is present, as does the protein level of at least one translational inhibition target. Furthermore, HopT-1 expression interferes with silencing induced by a transgene, indicating that it may function widely to inhibit Argonaute1 function. Consistent with this observation, lines overexpressing HopT-1 are smaller than wild-type plants, which may indicate pleiotropic effects due to general suppression of small RNAs. To what extent HopT-1 interferes with small RNA function, or the mechanism by which it suppresses Argonaute1, are yet to be determined.

Interaction between bacterial and viral infections

The most exciting implications of the work of Voinnet and colleagues [4] involve interactions between multiple pathogens on a single host. In natural conditions, organisms are bombarded by many potential microorganisms at one time. These pathogens may have neutral effects on one another, but more frequently they are antagonistic or synergistic. In some cases this may be due to the host's choice of defense strategy, as is the case in systemic acquired resistance, when recognition of a single biotrophic pathogen (a pathogen that takes nutrients from living tissue) enhances resistance to a broad range of other pathogens but leaves the host vulnerable to necrotrophic pathogens (a pathogen that takes nutrient from dead tissue) [12]. In other instances, pathogen-pathogen interactions may be more specific.

A role for small RNAs in virus resistance in plants has been known for some time [13,14]. Double-stranded RNA from viral replication intermediates or from secondary structures within the viral transcript is cleaved by host factors into small RNAs that target further degradation of viral transcripts and homologous sequences from the host. The proteins known as viral suppressors of silencing (VSRs) were discovered soon after, and it is likely that every plant virus encodes a VSR, with those from different viruses acting at different points in the RNA-mediated silencing pathway. In the case of viral resistance, the small virus-derived RNAs are the initial layer of defense, analogous with PTI, and the viral suppressor proteins are the counter-defense. VSRs can inhibit proteins that stabilize siRNA (for example, the VSR Hc-Pro), target the degradation of proteins that effect gene silencing (for example, the VSR P0), or bind siRNA duplexes (for example, the VSR P19) [6-9].

A well characterized example of a synergism that depends on VSRs is that between potato virus X (PVX) and the potyvirus potato virus Y (PVY). In this case, expression of the VSR Hc-Pro from PVY causes hyperaccumulation of PVX [15]. Interestingly, the synergism only occurs in one direction - PVY accumulates to the same level regardless of the presence of PVX, even though PVY also carries a VSR. Voinnet and colleagues [4] further expand our understanding of pathogen synergism by showing that infection with turnip mosaic virus, another potyvirus that encodes the VSR Hc-Pro, eliminates PTI against bacteria and allows 'non-host' bacterial pathogens to grow on *Arabidopsis*. How widespread this effect may be is unknown, but it provides insights into the challenge of protecting crops from pathogens.

Host-pathogen interactions are intricate and well balanced, as each organism tries to gain advantage in the battle for survival. As we learn that small RNAs are involved in nearly all biological processes, it is not surprising to discover that they are both weapons against pathogens and targets of pathogen attack. The work of Voinnet and colleagues [4] highlights the significance of miRNA-mediated resistance and the lengths to which bacteria have gone to suppress this resistance. The presence of suppressors of RNA silencing in both viruses and bacteria allows diverse pathogens to work together and defeat host defenses, and opens up the possibility that small RNA defenses and suppressors might be found in many more host-pathogen interactions.

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