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# Plant targets for *Pseudomonas syringae* type III effectors: Virulence targets or guarded decoys?

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# Plant targets for *Pseudomonas syringae* type III effectors: Virulence targets or guarded decoys?

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## Abstract

The phytopathogenic bacterium *Pseudomonas syringae* can suppress both pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) by the injection of type III effector (T3E) proteins into host cells. T3Es achieve immune suppression using a variety of strategies including interference with immune receptor signaling, blocking RNA pathways and vesicle trafficking, and altering organelle function. T3Es can be recognized directly or indirectly by resistance proteins monitoring specific T3E targets resulting in ETI. It is presently unclear whether the monitored targets represent *bona fide* virulence targets or guarded decoys. Extensive overlap between PTI and ETI signaling suggests that T3Es may suppress both pathways through common targets and by possessing multiple activities.

## Introduction

*Pseudomonas syringae* is a Gram-negative plant pathogenic bacterium whose strains have been classified into pathovars based on the host plant in which they were identified. *P. syringae* is a hemibiotroph and lives both on the surface and in the apoplast of the plant. In order to thrive in its host it must overcome the plant's innate immune response. This is accomplished in part by production of exopolysaccharides within the apoplast [1] and the production of compounds like coronatine or syringolin that alter plant responses [2,3]. However, many bacterial pathogens suppress immunity using type III effectors (T3Es) [4*, 5]. T3Es are proteins that are injected into host cells by a syringe-like apparatus called the type III protein secretion system (T3SS). The T3SS in plant pathogenic bacteria is called the Hrp T3SS because mutants in the corresponding genes are no longer able to elicit a hypersensitive response (HR), an immune-related form of programmed cell death, in non-host and resistant plants and are no longer pathogenic in host plants [6,7]. There are about 15–35 T3Es per *P. syringae* strain [8,9]. Those T3Es identified for their ability to elicit resistance were termed avirulence proteins (Avr), while T3Es subsequently identified in *P. syringae* are assigned a Hop (Hrp outer protein) nomenclature to generically indicate they are secreted by the Hrp T3SS. T3Es have various enzymatic activities including cysteine proteases (e.g., AvrPphB and AvrRpt2), mono-ADP-ribosyltransferases (HopU1 and HopF2), a phosphothreonine lyase (HopAI1), an E3 ligase (AvrPtoB), and a protein tyrosine phosphatase (HopAO1) [10]. These different activities can nevertheless lead to functional

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redundancy if the T3Es modify the same substrate using different mechanisms or act on separate parts of the same signaling pathways.

The plant immune system can be portrayed as two branches distinguished by their method of recognizing the invading microorganism. The first branch involves the recognition of pathogen (microbe)-associated molecular patterns (PAMPs), molecules highly conserved in microorganisms, and is termed PAMP-triggered immunity (PTI). PAMPs include molecules such as flagellin, EF-Tu, and chitin and PAMP recognition is mediated by immune receptor complexes containing plasma membrane-localized PAMP receptor-like kinases (RLKs) such as Flagellin-sensitive 2 (FLS2) for flagellin, the EF-Tu Receptor (EFR) for EF-Tu, and the Chitin Elicitor Receptor Kinase 1 (CERK1) for chitin [11,12](see Segonzac and Zipfel, this issue).

The other branch of the plant immune system recognizes effectors and is called effector-triggered immunity (ETI). Resistant plants can recognize individual bacterial T3Es using intracellular nucleotide-binding site, leucine-rich repeat (NB-LRR) resistance (R) proteins. According to the “guard hypothesis”, recognition is achieved by the R protein monitoring plant proteins for modification by T3Es and then responding by activating ETI [13]. The mechanism by which this occurs has been referred to as a ‘bait and switch’ as the molecule monitored can be viewed as bait that the R protein uses to detect the presence of a specific pathogen effector. The R protein itself acts as a molecular switch that is kept inactive by the bait until it is modified by the effector [14].

A variation of the “guard hypothesis” is the “decoy model”: here, the guarded protein is speculated not to be a virulence target but rather a decoy that mimics a virulence target of a pathogen effector [15,16**]. The differences between the two models lie primarily in whether or not the pathogen benefits from modifying/interacting with the guarded target. In this review, we refer to a host protein that is modified by a T3E as a T3E target. If it has been shown to benefit the pathogen then it is considered a virulence target. If there is data consistent with the “decoy model”, it is noted that it may be a guarded decoy. However, since the evidence for a T3E target being a guarded decoy is based on negative data, designating it as such is less certain.

Independent of the bacterial molecule recognized, both PTI and ETI appear to activate similar signaling pathways and immune responses, however, ETI generally activates them in a more prolonged and robust fashion than PTI and usually includes the HR [17]. The signaling pathways include MAP kinase (MAPK) cascades, calcium fluxes, transcriptional reprogramming and the alteration of hormone networks including the production of salicylic acid (SA). The plant immune responses include deposition of lignin and callose in the cell wall and production of reactive oxygen species and antimicrobial compounds [17,18].

This review focuses on how and where *P. syringae* T3Es act to suppress plant innate immunity. It highlights common nodes of PTI and ETI signaling that are targeted by various *P. syringae* T3Es. Additionally, this review also discusses how R protein complexes can indirectly recognize *P. syringae* T3Es and whether these R proteins are guarding decoys or virulence targets. There are several excellent recent reviews on related topics including reviews covering methods to assess the plant immune response [19], T3E inventories of different *P. syringae* strains [5], as well as reviews on plant pathogen T3Es activities and plant targets [10,20,21]. Much of the information on T3Es below is summarized in Figure 1 and Table 1.

## T3Es that target PAMP receptor-like kinase complexes

An effective site for T3Es to suppress innate immunity is PAMP recognition and several *P. syringae* T3Es target PAMP RLK complexes. Examples are AvrPto and AvrPtoB, T3Es that target plant kinases using different activities. AvrPtoB contains a C-terminal E3 ligase domain that ubiquitinates PAMP RLKs leading to their degradation [22**,23**] and AvrPto is a kinase inhibitor [16**] that inhibits PAMP RLK kinase activity. Therefore, AvrPto and AvrPtoB apparently achieve PTI suppression via interactions with PAMP RLKs (Fig. 1,Table 1). AvrPto and AvrPtoB were also shown to bind to the PAMP co-receptor, brassinosteroid associated kinase 1 (BAK1), and prevent its interaction with FLS2 [24*]. However, there is conflicting evidence for the interaction of AvrPto with BAK1 [25] and, therefore, it is unclear whether BAK1, the PAMP RLKs, or both represent the true virulence targets of these T3Es (see Segonzac and Zipfel, this issue).

## T3Es that target R protein complexes

The relatively broad specificity of some T3Es such as AvrPto, which targets multiple PAMP RLKs, may have allowed the plant to evolve a way to recognize its presence. AvrPto interacts with the Pto kinase inducing ETI. Pto may act as PAMP RLK decoy, as it is monitored (i.e., guarded) by the R protein Prf in tomato [26]. Alternatively, Pto may be a virulence target of AvrPto. The challenge of finding out whether Pto (and other potential decoys) is a *bona fide* decoy rests on whether the pathogen is more virulent in plants lacking the target. In the case of Pto, the virulence of *P. syringae* lacking AvrPto on tomato plants is similar whether Pto is present or absent. This suggests that Pto is not a ‘strong’ virulence target. Furthermore, AvrPto can enhance *P. syringae* virulence in plants lacking Pto probably due to its targeting PAMP RLKs [27,28]. Thus, Pto may act as a decoy, however, functional redundancy of Pto could mask its role in PTI or Pto could subtly impact the virulence of *P. syringae* in a manner that is difficult to detect using the bioassays currently available.

In addition to AvrPto, the Pto-Prf complex also recognizes AvrPtoB. Thus, a similar decoy scenario as above can be envisioned for AvrPtoB. However, AvrPtoB derivatives lacking the C-terminal E3 ligase domain are recognized by a complex of Prf and the Fen kinase (a kinase similar to Pto) [29]. Interestingly, full-length AvrPtoB ubiquitinates Fen targeting it for degradation via the 26S proteasome and preventing its recognition by Prf. Unlike Fen, the Pto kinase escapes ubiquitination by AvrPtoB because it inactivates the AvrPtoB E3 ligase by phosphorylation [30**]. The Pto and Fen kinases elegantly demonstrate that a T3E target can evolve to inactivate its corresponding T3E. If Pto and Fen are acting as guarded decoys then these decoys are certainly not of the run-of-the-mill variety. There is evidence that other Pto-like kinases can be part of Prf containing protein complexes which may increase the number of pathogen effectors that can be perceived by this immune receptor [31].

Another well established example of a monitored T3E target is RIN4 (RPM1-interacting protein 4), a plasma membrane-associated protein of *Arabidopsis thaliana* that resides in a complex with the R proteins RPM1 and RPS2. In a manner not well understood RPM1 perceives the phosphorylation of RIN4 upon its interaction with the T3Es AvrB and AvrRpm1. RPS2 recognizes the cleavage and subsequent elimination of RIN4 by the T3E AvrRpt2 [32]. The T3E HopF2 also targets RIN4 apparently without being detected by an R protein and suppresses ETI induced by AvrRpt2, but not by AvrB or AvrRpm1 [33*] (Fig. 1,Table 1). HopF2 is a mono-ADP-ribosyltransferase that can ADP-ribosylate RIN4 *in vitro* [34**]. HopF2 s modification of RIN4 may interfere with RIN4’s interaction with AvrRpt2. Therefore HopF2 suppresses ETI by preventing AvrRpt2 from cleaving RIN4 and being

detected by the R protein RPS2 [33*]. HopF2 also suppresses ETI induced by the T3E HopA1 but this is not known to be RIN4-dependant [4*,35]. Additional T3Es, including AvtPto, have been shown to interact with RIN4 [36]. RIN4 may therefore be a component of multiple R protein complexes.

Why is RIN4 targeted by multiple T3Es? The simple but vague answer is that it must play an important role in plant immunity. However, its role as a negative regulator of plant immunity [37] makes it a paradoxical target for T3Es. Recently, two plasma membrane $H^+$-ATPases, AHA1 and AHA2, were shown to be components of the RIN4 complex [38]. These ATPases are involved in stomatal opening and, therefore, it is possible that RIN4 is a virulence target that allows *P. syringae* to modulate stomatal opening. Additional evidence that RIN4 is a virulence target is that HopF2 promotes *P. syringae* growth in a RIN4-dependent manner [33*]. An alternative explanation is that RIN4 is a guarded decoy. Consistent with it being a guarded decoy, *P. syringae* virulence on Arabidopsis *rin4* mutant plants is not enhanced [39].

## T3E virulence targets identified using the logic of the “decoy model”

Even though the “decoy model” is inherently difficult to prove because it relies on negative data its logic was recently used to predict virulence targets for the T3E AvrPphB. AvrPphB is a cysteine protease that cleaves *avrPphB* susceptible 1 (PBS1) kinase and in a manner consistent with the “decoy model”, the R protein RPS5 detects this cleavage [40]. Also consistent with the “decoy model” is the fact that Arabidopsis *pbs1* mutants are not more susceptible to virulent strains of *P. syringae* [41]. If PBS1 is a guarded decoy, the true virulence targets of AvrPphB should be kinases similar to PBS1. Indeed, other PBS1-like kinases (PBLs) including *Botrytis*-induced kinase 1 (BIK1) are cleaved by AvrPphB (Table 1) [42**]. Two of these cleaved PBLs (BIK1 and PBL1) were shown to interact with FLS2 and be transiently phosphorylated upon FLS2 activation in a BAK1-dependent manner [42**,43]. Furthermore, plants lacking BIK1 and/or PBL1 were compromised in their PTI response to several PAMPs suggesting that at least these two targets of AvrPphB are important in PTI and are virulence targets of *P. syringae* (Fig. 1) [42**,43].

## T3Es targeting MAPK pathways

Early work in Arabidopsis showed that the MAPK cascade downstream of flagellin perception consisted of MEKK1, MKK4/MKK5 and MPK3/MPK6 [44]. Two T3Es have been shown to inactivate this cascade. One, HopF2, ADP-ribosylates and inhibits MKK5 preventing the phosphorylation of MPK3 and MPK6 in response to PAMP treatment [34**]. The other, HopAI1 (a phosphothreonine lyase) permanently deactivates MPK3 and MPK6 and other MAPKs by dephosphorylation [45,46] (Fig. 1,Table 1). A T3E that may also fit into this class is the protein tyrosine phosphatase HopAO1 [47,48]. MAPKs were thought to be putative targets of HopAO1 because transiently expressed HopAO1 in tobacco suppressed the HR induced by a constitutively active MAPK kinase [47]. However *in planta* expression of HopAO1, while capable of PTI suppression, did not inhibit the activation of MPK3 and MPK6 suggesting that they are not its direct targets [49]. Another T3E, AvrB, that induces the phosphorylation of RIN4 [50] also interacts with MPK4 and by an unknown mechanism enhances its activity [51]. These studies again emphasize the importance of MAPK signaling pathways as targets for T3E mediated PTI suppression. Analyzing the activity of MAPKs can help to identify where in innate immunity a particular T3E acts, as T3Es that prevent the activation of MAPKs are often found to target PAMP RLKs [52]. It was recently shown that four calcium-dependent protein kinases are involved in integrating signaling downstream of the PAMP RLKs [53] and these could potentially be modified by T3Es to suppress plant innate immunity.

## T3Es likely acting post-transcriptionally

To date there is no evidence that *P. syringae* T3Es can act as transcription factors to directly modulate gene expression as described for TAL effectors from *Xanthomonas* [54](see Scholze and Boch in this issue for an update on TAL effectors). However, some *P. syringae* T3Es appear to act post-transcriptionally. One of them, HopU1, was shown to be a mono-ADP-ribosyltransferase that ADP-ribosylates *in vitro* a glycine-rich RNA-binding protein (GRP7) and several other RNA recognition motif-containing RNA-binding proteins. Arabidopsis mutants lacking GRP7 are more susceptible to *P. syringae* compared to wild type plants and presumably HopU1 ADP-riboyslates GRP7 to interfere with its RNA-binding activity [55**]. Preliminary data suggest that GRP7 may regulate the translation of immunity-related products (Jeong and Alfano, unpublished data). In eukaryotes, RNA-binding proteins have been shown to act as a post--transcriptional control by determining whether specific mRNAs are translated [56]. It seems likely that HopU1 is targeting RNA-binding proteins to disable this regulation in response to biotic stress.

Another T3E that appears to function post-transcriptionally is HopM1, which targets several Arabidopsis proteins and initiates their degradation via the 26S proteasome [57**]. One of these proteins is MIN7, an ARF guanine nucleotide exchange factor, which is involved in vesicle trafficking [57**] (Fig. 1,Table 1). MIN7 was recently shown to localize to the trans-golgi network and early endosomes and play a role in mediating the internalization of constitutively cycling plasma membrane proteins [58]. Blocking vesicle trafficking could benefit the pathogen by inhibiting the movement of immunity-related compounds to the plasma membrane and cell wall/apoplast thereby limiting the plant immune response. Indeed, a knockout of MIN7 interferes with polarized callose deposition in response to the *P. syringae* Δ*cel* mutant that lacks HopM1 [57**]. Alternatively, the removal of MIN7 by HopM1 could prevent the recycling or mobilization of immunity-related plasma-membrane proteins (e.g., FLS2), which would limit the ability of plants to perceive or respond to the pathogen.

## T3Es that target plant organelles

Identifying T3E targets is critical for determining how they function as immune suppressors; however, knowledge of their site of action can also provide us with important clues. For instance, a number of T3Es that localize to the plant plasma membrane including AvrB, AvrRpm1, AvrPphB and AvrPto [59–61] target immune receptor complexes. Initial studies of *P. syringae* T3E inventories revealed that several T3Es have potential organelle targeting sequences [62]. Both chloroplasts and mitochondria have now been confirmed as subcellular locations for T3Es. The T3E HopI1 enters and remodels the chloroplast and reduces the production of the immunity-related plant hormone SA [63]. HopI1 contains a J domain that stimulates the ATPase activity of Hsp70 presumably helping to protect and re-fold host proteins. HopI1 causes Hsp70 to form large complexes in the chloroplast [64]. However, the link between Hsp70 and SA production needs to be determined. The T3E HopG1 localizes to mitochondria and although its mechanism of action and specific target are unknown at present it can alter respiration and basal levels of reactive oxygen species [65] (Fig. 1, Table 1). Further studies will likely identify additional T3Es that target these organelles. The study of these T3Es will help in the elucidation of the function of organelles in innate immunity.

## Concluding remarks

The *P. syringae* T3E research community has made much progress over the past several years in identifying T3E activities and targets. However, there is still much to do as the majority of T3Es activities/targets remain unknown and we do not understand how T3Es collectively disable plant innate immunity. The development of innovative biochemical, cell

biological, and bioinformatic techniques will be imperative to provide new insights into T3E function. It has become apparent that several T3Es have multiple plant targets and often contain multiple activities. Thus, we need careful target identification otherwise the elucidation of how T3Es suppress plant immunity will become very complex. At this moment, it seems clear that the "guard hypothesis" correctly predicts how R proteins can indirectly recognize *P. syringae* T3Es. From our perspective the "decoy model" represents a variation of the former model – whether the monitored protein is a virulence target or a decoy, it is guarded. Some virulence targets of T3Es may inadvertently be labeled as decoys based on the inability to identify a benefit to the pathogen that targets them. Nevertheless, in the case of AvrPto, AvrPtoB, and AvrPphB it seems likely that their guarded targets are decoys. Furthermore, this suggests that similar approaches to identify virulence targets can be taken with AvrRpt2 and other T3Es whose activity and guarded targets are known. It seems clear that by identifying virulence targets we will identify new components of plant immunity – components that may be unidentifiable by other approaches. In this fashion T3Es can be useful tools for plant biologists. And finally, with many effectors being identified from eukaryotic pathogens, it seems likely that they will have targets in common with prokaryotic pathogens. Therefore, *P. syringae* T3Es and those from other well studied bacterial pathogens represent model systems to explore plant immunity.

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**Figure 1. The site of action of *Pseudomonas syringae* T3Es**

*P. syringae* injects type III effectors (T3Es) (red text) into the host cell using a type III secretion system (T3SS). *P. syringae* PAMPs are recognized by the plant using PAMP RLKs such as FLS2 (light blue) leading to PAMP-triggered immunity (PTI). PTI includes MAP kinase and calcium-dependant protein kinases (CDPKs) signaling leading to the production of reactive oxygen species (ROS) by NADPH oxidases (grey box); accumulation of hormones such as salicylic acid (SA); and alterations in immunity-related transcription and cell wall defenses. T3Es suppress PTI at the level of the PAMP receptor complexes with AvrPto and AvrPtoB targeting the PAMP RLKs themselves including FLS2 and its co-receptor BAK1 (dark blue), while the T3E AvrPphB cleaves the PAMP RLK-associated kinase BIK1 (pink hexagon). HopF2 and HopAI1 inactivate the MAPK cascade by ADP-ribosylating MAP kinase kinases (MAPKK) and dephosphorylating MAP kinases (MAPK), respectively. The T3E HopM1 induces the degradation of the ARF-GEF MIN7 interfering with vesicle trafficking. The T3E HopU1 ADP-ribosylates RNA-binding proteins such as GRP7, likely inhibiting RNA translation. T3Es also target organelles with HopG1 targeting the mitochondria and HopI1 the chloroplasts where it activates Hsp70 and suppresses SA production. The R protein RPS2 (green) recognizes the cleavage of RIN4 by the T3E AvrRpt2 but the T3E HopF2, which ADP-ribosylates RIN4, can prevent this recognition. The R protein RPM1 (turquoise) recognizes hyperphosphorylation of RIN4 induced by the T3Es AvrB and AvrRpm1. AvrB also interacts with RAR1 and MAP kinase 4 (MPK4) that form a complex with RIN4. T3E recognition by R proteins leads to effector-triggered immunity (ETI) that largely overlaps with PTI. See text for additional details.

**Table 1**

Activity, plant targets and localization of *Pseudomonas syringae* type III effectors.

| T3E | Pathovar | Activity | Target(s) | Subcellular Localization[a] | Reference |
|---|---|---|---|---|---|
| AvrB | *glycinea* race 0 | Unknown | RIN4/RAR1/MPK4 | Plasma membrane | [50,51,59] |
| AvrPphB | *phaseolicola* race3 | Cysteine protease | PBS1/BIK1/PBLs | Plasma membrane | [42**,59,61,66,67] |
| AvrPto | *tomato* JL1065 | Kinase inhibitor | Pto/Fen/PAMP RLKs/BAK1 | Plasma membrane | [16,24*,25,36,60] |
| AvrPtoB | *tomato* DC3000 | E3 ubiquitin ligase | Pto/Fen/PAMP RLKs/BAK1 | Non-discrete | [22**–24*,36,68] |
| AvrRpm1 | *maculicola* M6 | Unknown | RIN4 | Plasma membrane | [50,59] |
| AvrRpt2 | *tomato* JL1065 | Cysteine protease | RIN4 | Non-discrete | [69,70] |
| HopAI1 | *tomato* DC3000 | Phosphothreonine lyase | MPK3/MPK6 and other MPKs | Unknown | [46] |
| HopAO1 | *tomato* DC3000 | Protein tyrosine phosphatase | Unknown | Non-discrete | [47,48] |
| HopI1 | *maculicola* ES4326 | J domain protein | Hsp70 | Chloroplast | [63,64] |
| HopF2 | *tomato* DC3000 | Mono-ADP-ribosyltransferase | RIN4/MKK5 | Plasma membrane | [33*,34**,71] |
| HopG1 | *tomato* DC3000 | Unknown | Unknown | Mitochondria | [65] |
| HopM1 | *tomato* DC3000 | Unknown | MIN7 and others | Endomembrane | [57**] |
| HopU1 | *tomato* DC3000 | Mono-ADP-ribosyltransferase | GRP7 and other RNA-binding proteins | Non-discrete | [55**] |
| HopZ1 | *syringae* A2 | Cysteine protease/Acetyltransferase? | Unknown | Plasma membrane | [72–74] |
| HopZ2 | *pisi* 895A | Cysteine protease/Acetyltransferase? | Unknown | Plasma membrane | [72–74] |
| HopZ3 | *syringae* B728a | Cysteine protease/Acetyltransferase? | Unknown | Non-discrete | [72–74] |

[a]Localization *in planta*. We refer to the localization as ‘non-discrete’ when the protein was not shown to localize to a specific site inside the plant cell.