

TYPE III SECRETION SYSTEM EFFECTOR PROTEINS: Double Agents in Bacterial Disease and Plant Defense

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■ **Abstract** Many phytopathogenic bacteria inject virulence effector proteins into plant cells via a Hrp type III secretion system (TTSS). Without the TTSS, these pathogens cannot defeat basal defenses, grow in plants, produce disease lesions in hosts, or elicit the hypersensitive response (HR) in nonhosts. Pathogen genome projects employing bioinformatic methods to identify TTSS Hrp regulon promoters and TTSS pathway targeting signals suggest that phytopathogenic *Pseudomonas*, *Xanthomonas*, and *Ralstonia* spp. harbor large arsenals of effectors. The Hrp TTSS employs customized cytoplasmic chaperones, conserved export components in the bacterial envelope (also used by the TTSS of animal pathogens), and a more specialized set of TTSS-secreted proteins to deliver effectors across the plant cell wall and plasma membrane. Many effectors can act as molecular double agents that betray the pathogen to plant defenses in some interactions and suppress host defenses in others. Investigations of the functions of effectors within plant cells have demonstrated the plasma membrane and nucleus as subcellular sites for several effectors, revealed some effectors to possess cysteine protease or protein tyrosine phosphatase activity, and provided new clues to the coevolution of bacterium-plant interactions.

INTRODUCTION

Many Gram-negative pathogens of plants and animals possess type III secretion systems (TTSS) that can inject bacterial virulence “effector” proteins into host cells (45). The TTSS is important to the pathogenicity of phytopathogens in the genera *Pseudomonas*, *Xanthomonas*, *Ralstonia*, *Erwinia*, and *Pantoea*, bacteria that colonize the intercellular spaces (apoplast) of plants and are generally capable of eliciting plant cell death at some stage in pathogenesis. Most of these pathogens are host specific and have compatible interactions leading to virulence in a limited range of susceptible plant species or cultivars of a species. In resistant

plants, an incompatible interaction develops, which is marked by the hypersensitive response (HR), a rapid, programmed death of plant cells that occurs at the site of infection and is associated with pathogen avirulence. As discussed below, pathogenesis involves the suppression of basal plant defenses against microbes, evasion or suppression of pathogen-specific HR defense surveillance, parasitic growth in the apoplast, and eventual production of lesions and other characteristic disease symptoms. TTSS mutants fail at the first step in this sequence, and the particular collection of TTSS effectors produced by a strain also are likely to determine compatibility and subsequent patterns of pathogen growth and symptom production. Thus, TTSS effectors are central to the pathogenicity of these bacteria, but how many TTSS effectors do phytopathogens produce? How are these diverse proteins translocated across plant cell walls and plasma membranes? What cellular functions or molecular activities are performed by effectors within host cells? How is the TTSS effector system integrated with other factors involved in plant interactions? These issues are broadly important in molecular plant pathology, and they have attracted far more research than can be covered here. Thus, our review is limited to recent work and examples that are particularly illustrative of the exciting progress that has been made.

We must first explain several terms associated with phytopathogen TTSS effector systems. The TTSS pathway is encoded by *hrp* (HR and pathogenicity) and *hrc* (HR and conserved) genes (23). The Hrc proteins direct secretion of TTSS substrates across the bacterial envelope, whereas a subset of the Hrp proteins (only partially defined) are themselves secreted by the TTSS and direct the translocation of effectors through host cell barriers. The term “effector” denotes the subset of TTSS-substrates that function primarily inside host cells, but some proteins secreted by the TTSS of animal pathogens appear to have multiple roles in promoting effector translocation and acting directly in host cells (45). Because of this ambiguity, we address all proteins shown to travel the Hrp TTSS in this review, including those thought to function primarily as helpers in the delivery of true effectors. Several of the best-studied TTSS effectors are designated as Avr proteins because they were detected through gain-of-function avirulence phenotypes (86, 145). Many more effectors subsequently have been identified by their ability to travel the TTSS pathway, and these are designated Hop (Hrp outer protein) in *Pseudomonas* (4), Xop (Xanthomonas outer protein) in *Xanthomonas* (118), or Pop (*Pseudomonas* outer protein, as based on a previous genus designation) in *Ralstonia* (9).

Terms for different types of resistance also warrant explanation. These are best explained with an example. *Pseudomonas syringae* pv. *glycinea* is generally virulent on soybean, but soybean cultivar Harosoy, which carries the *Rpg1* R gene shows race-specific resistance to race 1 of *P. syringae* pv. *glycinea*, which carries the cognate *avrB* gene (144). In contrast, all cultivars of soybean show nonhost resistance to *P. syringae* pv. *tomato*, a pathogen of tomato. Both race-specific and nonhost resistance involve elicitation of the HR, and soybean plants can recognize several effectors produced by *P. syringae* pv. *tomato* (94). Whether R protein

surveillance of effectors is the primary determinant of nonhost resistance is unclear (103, 154), but similar examples can be found with many pathovars of *P. syringae* and *X. campestris* and affiliated species.

Importantly, plants are universally resistant to nonpathogens, such as *Escherichia coli* and *Pseudomonas fluorescens*, as well as to most TTSS-deficient phytopathogen mutants. Nonpathogenic bacteria elicit a defense response not involving the HR, which is variously known as localized induced resistance (51, 93), innate immunity (66, 119), or basal resistance (115). We use the term basal resistance here. As explained below, we postulate that basal defenses must be suppressed by TTSS effectors before the interaction can progress to either nonhost resistance, race-specific resistance, or compatibility, depending on the plant-bacterium combination. A theme of this review is that many of the proteins delivered by the TTSS act as double agents that both elicit and suppress plant defenses.

BASAL AND HR DEFENSES AND PLANT CELL DEATH

To better understand the functions of TTSS effectors, we must further explain basal and HR defenses. TTSS effectors presumably promote bacterial growth in the apoplast by defeating host defenses and releasing nutrients from plant cells. We can presently distinguish two levels of host defenses that must be defeated. Basal defenses are triggered from the outside of plant cells by nonpathogens, TTSS mutants, heat-killed bacteria, and factors bearing “pathogen-associated molecular patterns” (PAMPs) (66, 111, 119). These factors are also known as general elicitors, and they represent universal microbial molecules (not pathogen-specific, despite the term PAMP), such as lipopolysaccharide (51), flagellin (66), and cold shock protein (57). Basal resistance appears to be highly localized (hence the alternative term local induced resistance), and many of the same microbial molecules elicit innate immunity in animal cells (111). Basal resistance to nonpathogenic bacteria is manifested in the formation of a callose-rich papilla beneath the plant cell wall at the site of bacterial contact, localized production of reactive oxygen species, and increased expression of phenylpropanoid pathway genes and of other defense-associated genes (84). Importantly, nonpathogens and PAMPs typically do not elicit the HR, although an exception involving flagellin is discussed below.

In contrast, the HR is elicited inside of plant cells by many phytopathogen effectors during “gene-for-gene” interactions involving matching *avr* and R genes (86). The molecular recognition and signal transduction events leading to programmed cell death and the defense of surrounding plant tissues are unexpectedly complex given the two-component genetics underlying race-specific, incompatible interactions. This phenomenon has been the focus of most research on TTSS effectors, as recently reviewed (109, 115).

Little is known about the cell death associated with the lesions that develop in later stages of compatible interactions involving virtually all pathogens with a TTSS. In a seminal review of pioneering physiological studies on the HR, Klement

(91) suggested that “the development of hypersensitive and normosensitive necrosis must be considered as being similar processes; their induction however requires different numbers of bacterial cells and their development proceeds at different rates.” Supporting this concept, a recent large-scale mRNA expression profile analysis of *Arabidopsis* responses to compatible and incompatible strains of *P. syringae* comprehensively documents the overall similarity of the responses, which differ primarily in their timing and suggests that a common signal transduction pathway underlies both (158). In this regard, it is intriguing that plants compromised in the capacity to undergo programmed cell death are unexpectedly more resistant to TTSS-dependent phytopathogens in compatible interactions (101, 148).

The multifaceted role of bacterial flagellin in defense elicitation requires further discussion. A conserved domain of approximately 22 amino acids (flg22) in the flagellin proteins of diverse bacterial species elicits defense responses associated with basal resistance (58). Defense elicitation by the flg22 peptide in *Arabidopsis* is dependent on FLS2, a receptor-like kinase with an extracellular leucine-rich repeat domain, which activates a MAP kinase cascade that increases basal defenses (12, 65). However, the flagellins of *P. syringae* pv. *tomato* and *P. syringae* pv. *glycinea* can instead elicit the HR in tobacco unless posttranslationally modified by a *P. syringae* pv. *tabaci* glycosylation system during heterologous expression (155, 156). Surprisingly, a flagellin-deficient mutant of *P. syringae* pv. *tabaci* no longer elicits the HR in nonhost tomato and instead is able to grow and produce lesions similarly to the compatible *P. syringae* pv. *tomato* (141). These observations suggest that plants may recognize multiple features of flagellins and that defensive recognition of flagellin can be a primary determinant of host range in some pathosystems.

TTSS EFFECTORS: FUNCTIONAL AND COMPARATIVE GENOMICS

Identifying TTSS Effector Genes in Sequenced Phytopathogen Genomes

Genome sequencing projects now enable comprehensive identification of TTSS effector genes in individual phytopathogen strains. This is noteworthy because virulence phenotypes of most effectors are too subtle (apparently because of redundancy) to permit identification in collections of random transposon mutants. Thus, identification by avirulence assays involving test plants carrying cognate R genes has been the historical approach. Genomic sequencing facilitates comprehensive searches based on Hrp promoter activity, on translocation of transposon-generated reporter protein fusions into plant cells, or on sequence patterns associated with Hrp promoters and TTSS targeting domains (33, 41, 67, 80).

Many of the Hrp effector genes are induced in *Xanthomonas* and *Ralstonia* by an AraC-type transcriptional activator that is activated by a two-component regulator,

or in *Pseudomonas*, *Erwinia*, and *Pantoea* by an ECF-type alternative sigma factor that is activated by σ^{54} -enhancer binding proteins (4, 38, 112). Genes controlled by these factors have been globally identified by cDNA-AFLP (118), reporter transposons (59, 104), or as part of in vitro expression technology (IVET) studies (22). Alternatively, candidate members of Hrp regulons have been identified on the basis of predicted promoter sequences in the complete genomes of *R. solanacearum* GMI1000 (135), *X. campestris* pv. *campestris*, *X. axonopodis* pv. *citri* (46), and *P. syringae* pv. *tomato* DC3000 (59, 179). These studies have revealed many genes in each Hrp regulon (in addition to the TTSS machinery genes), but not all of these genes encode TTSS effectors, and some TTSS effector genes are not regulated by the Hrp system.

Recognition of sequence patterns associated with targeting to the TTSS pathway and assays for Hrp-dependent secretion or translocation provide a more direct method for identification of effector genes. TTSS effectors have a modular structure, and targeting information resides in the first 100–150 amino acids (45). The first 15 mRNA codons or amino acids of the *P. syringae* effector AvrPto are essential, and they may be sufficient for weak translocation of a Cya (adenylate cyclase) reporter (7, 136). The remaining N-proximal amino acids contain supplementary targeting information, such that the first 50 amino acids are sufficient for robust secretion or translocation of adenylate cyclase or truncated AvrRpt2 avirulence domain reporters, but maximal secretion or translocation requires the first 100 amino acids (34, 114, 136).

As discussed below, the secretion/translocation of some effectors is enhanced by customized chaperones, which are expected to interact somewhere between residues 16–150 of effectors (5, 56). Typical *P. syringae* effectors have an hydrophobic amino acid in position 3 or 4, no acidic amino acids in the first 12 positions, and a bias for serine and proline in the first 50 positions (41, 67, 70, 128). The use of these patterns, along with Hrp promoter sequences, low %G + C content, and other features associated with effector genes, permits facile bioinformatic identification of effector candidates in *P. syringae* genome sequences. However, at least one *P. syringae* pv. *tomato* DC3000 effector that lacks these patterns is nevertheless translocated into plant cells by the TTSS, and several *Xanthomonas* and *Ralstonia* effectors do not show these patterns at all (128, 136, 167). Thus, even in well-studied strains such as *P. syringae* pv. *tomato* DC3000, we cannot yet say that all effector genes have been found.

Nevertheless, over 150 effector genes have been identified collectively in various *P. syringae* strains, and more than 40 TTSS substrates have been experimentally confirmed in the model strain DC3000 (28, 136, 167). Because of the deluge of information on these proteins, frequently updated, web-based resources are becoming increasingly important in the analysis of the role of effectors in pathogenesis. As one example, <http://pseudomonas-syringae.org> contains a database on *P. syringae* pv. *tomato* DC3000 TTSS substrates and candidates. Included is information on more than 35 features of these proteins, ranging from nomenclature to biochemical activity, as well as links to related databases and other tools for

functional genomics and genome viewing. Importantly, genomic-based effector inventories are providing new insights into the evolution of the TTSS effector system, and they reveal additional patterns associated with subcellular locations in host cells and the activities of effector families.

TTSS Effector Genes in a Genomic Context

TTSS effectors are commonly associated with mobile genetic elements, and many appear to have been acquired by horizontal gene transfer (11, 87). For example, the *P. syringae* effector genes are associated with regions missing in the related bacteria *P. aeruginosa* and *P. putida*, and some are carried on plasmids or in hypervariable regions of the genome, such as the exchangeable effector locus (3, 28, 36, 48). In all genomes that have been sequenced to date, the *hrp/hrc* genes are found clustered in a single region of the chromosome, or on a 2.1-Mb megaplasmid in the case of *R. solanacearum* or a 150-kb plasmid in *Pantoea agglomerans* (*Erwinia herbicola*) pv. *gypsophilae* (33, 107). These clusters of *hrp/hrc* genes are typically flanked by regions that contain different effector genes in different bacterial species or pathovars (3, 33). The exchangeable effector locus in *P. syringae* is one such hypervariable region, and another apparent hot spot for effector gene recombination has been identified elsewhere in the *P. syringae* genome (3, 10). The use of primers to conserved sequences flanking these hot spots provides another approach to isolating effector genes (10, 36, 48).

Duplications within genomes of effector genes and associated mobile genetic elements may support ongoing insertions and deletions of effector genes. For example, *P. syringae* pv. *maculicola* M6 carries two copies of *avrPphE*. One is on a plasmid, and the other is on the chromosome and is a site for integration of the plasmid, which carries at least one other effector gene (133). Loss of gene function also may be important in the evolution of virulence (124). Several candidate effector genes in DC3000 are disrupted by mobile genetic elements (28, 67); the truncated product of two such genes can be translocated into plant cells by the *P. syringae* TTSS (136); frameshifted effector pseudogenes have been found in the exchangeable effector loci of several *P. syringae* pathovars (36, 48); and many effector genes have limited distribution among pathovars and races of *P. syringae* and *X. campestris* and affiliated species.

The taxonomic distribution patterns of effector genes have yielded only tantalizing hints regarding the determination of host and tissue specificities. For example, comparison of the known effectors in *X. campestris* pv. *campestris* (a xylem-invading pathogen of crucifers) and *X. axonopodis* pv. *citri* (a mesophyll-limited pathogen of citrus) revealed the striking absence of members of the AvrBs3 family in *X. campestris* pv. *campestris* (46). Members of this effector family are found in several other xanthomonads and in *R. solanacearum* (but not in *P. syringae*) (28, 96, 135). There are other interesting distribution patterns involving well-studied effectors. AvrBs2 homologs are widespread in *X. campestris* pathovars and affiliated

species, but they are absent from *P. syringae*, whereas the opposite is observed with AvrE. In contrast, members of the AvrPphE, AvrPphF, and AvrRxv/YopJ families are widespread in both groups, and the latter family is also found in many animal pathogens (28, 46, 96).

TTSS effectors are likely to be important determinants of host specificity in the *P. syringae* and *X. campestris* groups. However, the TTSS machinery itself may have little role in host specificity, as indicated by the ability of a *P. syringae* pv. *tomato* Δ *hrp/hrc* mutant expressing the *hrp/hrc* gene cluster of a bean pathogenic *P. syringae* pv. *syringae* strain to retain normal virulence on tomato and avirulence on bean (60). The relationship between effector inventories and host range in *P. syringae* is still obscure. For example, analysis of the presence or absence of 12 TTSS effectors in 60 *P. syringae* strains from various pathovars revealed no correlation with the host of isolation (69). One explanation for this observation is that allelic variations in effectors that are present in many (or all) pathovars are important determinants of host specificity (147). Importantly, an example of host range limitation by a single TTSS effector has recently been reported: PthG in *Pantoea agglomerans* pv. *gypsophylae* appears to be the sole factor preventing this gypsophila pathogen from expanding its host range to beet (55).

TTSS EFFECTOR DELIVERY: SPECIALIZATIONS FOR PHYTOPATHOGENS

Cytoplasmic TTSS Chaperones

TTSSs in animal pathogens are dependent on molecular chaperones for the secretion of a subset of secreted proteins (56, 125, 165). Chaperones share little sequence similarity with each other, but they have the general characteristics of being small (circa 15 kDa), acidic (isoelectric points <6), and with a predicted amphipathic region in their C termini. Also, their encoding gene is often next to the cognate effector gene (166). TTSS chaperones have been separated into classes based on whether they interact with one effector or multiple effectors (or other TTSS-secreted proteins) and whether they function in pathogenic TTSSs or in the flagellar biogenesis TTSS (42, 125). TTSS chaperones act in the cytoplasm to stabilize or prevent association of effectors before delivery (123), to maintain effectors in a secretion competent nonglobular state (146), and in some cases to regulate the TTSS (61). The role of chaperones in targeting effectors to the TTSS pathway is still unresolved, and the chaperone-binding domain within a secreted protein may constitute a second secretion signal (40), which acts independently of signals in the first 15 amino acids or mRNA codons of the effector (8, 143). TTSS chaperones in *Salmonella typhimurium* were shown to provide specificity to effector secretion signals, such that in the absence of cognate chaperones, effectors were inappropriately secreted through the flagellar TTSS (99). Importantly, TTSS chaperones may affect the order of secretion of TTSS effectors, allowing a hierarchy of effector delivery (25, 159). Indeed, in the *Yersinia* TTSS, the effectors that

are predicted to be needed early in pathogenesis to inhibit phagocytosis utilize chaperones for their secretion (43, 56).

TTSS chaperones in phytopathogens were demonstrated only recently, partly because many of the effectors identified by gain-of-function avirulence phenotypes are not associated with chaperones. ShcA (specific Hop chaperone) from *P. syringae* pv. *syringae* 61 assists in the secretion of the HopPsyA effector (163). Similarly, the *E. amylovora* DspB/F chaperone is required for secretion of DspA/E (64). Several other candidate *P. syringae* type III chaperones have been identified (28, 163, 167). Three of these candidate chaperones, ShcM, ShcV, and ShcF, recently have been confirmed to act as chaperones for the effectors HopPtoM, HopPtoV, and HopPtoF, respectively (17, 137, 167). Moreover, database searches and genome projects have identified several other candidate chaperones in *P. syringae* (28, 70, 163, 167). TTSS chaperones have yet to be demonstrated in *R. solanacearum*, *X. campestris*, or *X. axonopodis*, although genome sequences are available (46, 135). Because there is evidence that chaperones help effectors compete for access to TTSSs, the identification of phytopathogen chaperones may reveal which effectors are translocated first into the plant cell and thus lead to a better understanding of the early stages of plant pathogenesis.

Extracellular TTSS Pathway Proteins

The Hrc proteins enabling effector transport across the bacterial envelope are broadly conserved in the TTSSs of plant and animal pathogens and in the flagellar biogenesis system (21). In contrast, extracellular components of the TTSS machinery are more specialized and variable because of their interactions with host cell barriers. However, these extracellular proteins appear to perform similar functions in all phytopathogens and can be divided into three classes: Hrp pilus subunits, harpins, and translocator factors (the latter defined here as forming a TTSS delivery pore in the host plasma membrane) (30). The pilus proteins and harpins are secreted in particular abundance by phytopathogen TTSSs in culture. All phytopathogens appear to produce a Hrp pilus, which serves as a conduit for effector delivery, as recently reviewed (72). The narrow external diameter (8 nm) of the Hrp pilus is consistent with its expected role in delivering effectors through the plant cell wall matrix and suggests that most effectors enter the host cytoplasm in an unfolded state.

Harpins have yet to be shown to have any role in effector delivery, and they have been studied primarily for their ability to elicit plant defenses. However, many properties of harpins point to an interaction with the plant cell wall and plasma membrane, which invites the hypothesis that they are helpers in effector delivery. Harpins differ from other proteins secreted via the Hrp TTSS by their defining properties of being glycine-rich, cysteine-lacking, and possessing heat-stable HR elicitor activity when infiltrated at relatively high concentrations into the leaf apoplast of tobacco and several other plants. Importantly, harpins differ from true effectors in showing no apparent relationship between elicitor activity

and host range, and they elicit the HR from the outside rather than the inside of plant cells (129, 157).

Harpin-like proteins have been reported in all genera of phytopathogens that have a TTSS (2, 4). These proteins include the Hpa1/HpaG family in *Xanthomonas* (176), which was recently shown to have HR elicitor activity (89). Some pathogens produce multiple harpins. For example, *Erwinia amylovora* and *P. syringae* were previously shown to produce two harpins each, HrpN/HrpW and HrpZ/HrpW, respectively (37, 88). HrpW is the prototype for a subclass of harpins that have an apparently bifunctional, two-domain structure. The N-terminal domain is particularly glycine-rich, whereas the C-terminal domain shows similarity to pathogen pectate lyases (37, 88). Analysis of the complete genome sequence of *P. syringae* pv. *tomato* DC3000 revealed the presence of two additional harpins with a two-domain structure similar to that of HrpW (28). The C-terminal domains of these proteins, HopPtoP and HopPmaH_{Pto}, are similar to lytic transglycosylases and pectin lyases, respectively, and both show heat-stable HR elicitor activity when infiltrated into tobacco (A.R. Ramos & A. Collmer, unpublished).

The similarities of HrpW and HopPmaH_{Pto} with pectic enzymes, and the observed ability of HrpW to bind specifically to pectate lyase (37), argue for a site of action in the plant cell wall. Furthermore, the higher conservation of the pectate lyase domain among HrpW homologs suggests that this domain has a biologically important interaction with pectic polymers (35), which notably control porosity of the cell wall (18). Harpins are secreted through the Hrp pilus and accumulate along the length of the growing structure (82, 83, 100), which would be consistent with a role in assisting pilus penetration through the cell wall matrix or in forming a pore upon pilus contact with the plasma membrane. Indeed, purified HrpZ harpin of *P. syringae* pv. *phaseolicola* can stably associate with liposomes and form pores in synthetic lipid bilayers, which suggests such a role in translocation of effectors across the plasma membrane (98).

The *E. amylovora* HrpN harpin elicits defenses through the systemic acquired resistance pathway (149, 168), and its activity is dependent on salicylic acid, NPR1/NIM1, NDR1, and EDS1 (50, 127), which suggests that harpins elicit plant defenses through the same signal transduction pathways that are activated by Avr effectors (with the latter differing among each other regarding requirements for NDR1 and EDS1). A consequent puzzle is why harpins produced by biotrophic pathogens, such as *E. amylovora*, *P. syringae*, and *R. solanacearum*, do not trigger the HR and avirulence during pathogenesis, given that their hosts are hypersensitive to the harpins they produce (129). The explanation may reside in dosage or in suppression of HR defenses by effectors that are injected into host cells. Evidence for either explanation can be found in the delayed expression of the PopA harpin-like protein by wild-type *R. solanacearum* during infection and the avirulence triggered by engineered strains that express PopA constitutively (85). Similarly, the responses of *Nicotiana benthamiana* leaves to the differing levels of the *P. syringae* pv. *phaseolicola* HrpZ harpin produced by bacteria, by transgenic plants, or by PVX expressing harpin with a signal peptide suggest that levels far

higher than that produced by bacteria during infection are needed to elicit the HR (157). Although direct comparisons are difficult because harpins and injected Avr effectors operate outside and inside of plant cells, respectively, the latter proteins appear to trigger the HR at far lower levels and clearly can do so during natural infections.

The translocation of TTSS effector proteins into host cells requires TTSS-secreted accessory proteins called translocators, which form pores in the eukaryotic plasma membrane. These have been extensively characterized in several animal pathogens (30, 45). Less is known about TTSS translocators in plant pathogens. However, recent evidence suggests that HrpF acts as a translocator in *X. campestris* (32, 134). HrpF shares similarity with *P. syringae* HrpK, *Rhizobium* NolX, and *R. solanacearum* PopF1/F2, and *P. syringae* *hrpK* mutants have phenotypes consistent with this protein functioning as a translocator in the *P. syringae* TTSS (T. Petnicki-Ocwieja, K. van Dijk & J.R. Alfano, unpublished). Clearly, much more research is needed to understand how these proteins interact with other components of the TTSS, how they promote effector translocation across the host plasma membrane, and how plants defend themselves against this prokaryotic violation. In this regard, it is noteworthy that the *Yersinia* YopB translocator factor can elicit innate immune defenses in epithelial cells unless these responses are suppressed by multiple injected Yop effectors (164).

TTSS EFFECTOR FUNCTIONS

Effectors and Suppression of Plant Defenses

The Hrp systems of *P. syringae* and *X. campestris* were implicated in the suppression of basal defenses long before these systems were shown to deliver effector proteins. Lindgren and colleagues showed that defense-related transcripts were induced in bean by avirulent *P. syringae*, saprophytes, and a *P. syringae* *hrp* mutant, but not by virulent Hrp-proficient *P. syringae*, which suggested that the Hrp system suppressed basal defenses (77, 78). Mansfield and colleagues then showed that saprophytes and *hrp* mutant pathogens induced regions of thickened plant cell wall called papillae at contact points between bacterial cells and the plant cell, which were not induced by virulent wild-type pathogens (20, 27).

There were also early clues that some Avr proteins might suppress, as well as elicit, HR defenses. For example, coexpression of cloned *avrRpt2* and *avrRpm1* genes in *P. syringae* pv. *tomato* DC3000 or *P. syringae* pv. *maculicola* M4 revealed that AvrRpt2 was able to interfere with the elicitation of the characteristically fast HR that is normally elicited by AvrRpm1 (in contrast to the slower HR elicited by AvrRpt2) in *Arabidopsis* ecotype Columbia (131, 132). The concept that some effectors might be HR suppressors was crystallized by the discovery that effector gene mutations in *P. syringae* pv. *phaseolicola* “unmasked” cryptic Avr activity within a virulent strain, suggesting that the disrupted effector suppressed an HR that would otherwise be triggered by one or more of the effectors in that strain

(76, 160). These papers provided evidence that AvrPphF, AvrPphC, and VirPphA blocked the HR, but they left open the possibility that the suppressors were acting in the bacterial cell by altering gene expression or effector access to the TTSS (76, 160). Suppressor activity inside plant cells was established with the demonstration that AvrPtoB (in the same effector family as VirPphA) could suppress HR processes dependent on two R genes, *Pto* and *Cf9*, when delivered with an *Agrobacterium*-mediated transient expression system in *N. benthamiana* (1). Figure 1 presents a conceptual overview of TTSS effectors functioning as both elicitors and suppressors of plant defenses.

Activated plant defense systems express many physiological responses, including the HR, pathogenesis-related (PR) gene expression, phytoalexin production, generation of reactive oxygen and nitrogen species, and callose deposition in the cell wall (47). HR assays are particularly easy to score and have revealed that the *P. syringae* pv. *tomato* DC3000 effectors AvrPphE_{Pto}, AvrPpiB1_{Pto}, AvrPtoB, AvrRpt2, HopPtoD2, HopPtoE, HopPtoF (an AvrPphF homolog), and HopPtoN can suppress an HR induced by other test Avr proteins (1, 14, 26, 53, 79, 102, 105). These observations suggest that approximately one third of the effectors in DC3000 have HR suppressive activity, and the use of *Agrobacterium* or PVX-mediated transient expression systems and mixed inoculum involving two TTSS-proficient bacteria in various experiments demonstrated that suppression in all cases tested is occurring inside plant cells (53, 79). Interestingly, several of the effectors are able to suppress a programmed cell death response induced by the pro-apoptotic mouse protein Bax and other elicitors in yeast (1, 79). The ability of these effectors to suppress different types of programmed cell death in different organisms suggests that they act on conserved pathways.

Do the effectors that suppress the HR suppress other markers of plant defense? At least in some cases, TTSS effectors appear to be suppressing PR genes (39, 79) and the oxidative burst (26). Notably, HopPtoD2 suppresses a nonhost HR and an HR-like response that is induced by transient expression of an active MAP kinase kinase (MAPKK) in tobacco. The latter is known to be involved in defense pathways, thus suggesting that HopPtoD2 acts downstream of this MAPKK (53). This is interesting because this MAPK pathway has been shown to be activated in response to a number of elicitors of defense responses, and a homologous MAPK pathway in *Arabidopsis* is activated upon recognition of the flagellin PAMP (12, 174). These observations suggest that HopPtoD2 acts at a point of convergence in defense signal transduction pathways to suppress both host-specific defenses triggered by Avr proteins and basal defenses triggered by PAMPs. Importantly, the *P. syringae* effectors, AvrPto and AvrRpt2, when expressed in transgenic *Arabidopsis*, supported increased growth of TTSS-defective *P. syringae* mutants (39, 71). Moreover, transgenic plants expressing AvrPto had reduced callose deposition and expression of genes encoding secreted or defense-related proteins when challenged with a TTSS-defective mutant compared to wild type *Arabidopsis* (71). These phenotypes suggest that AvrPto and AvrRpt2 have the ability to suppress PAMP-triggered basal defenses.

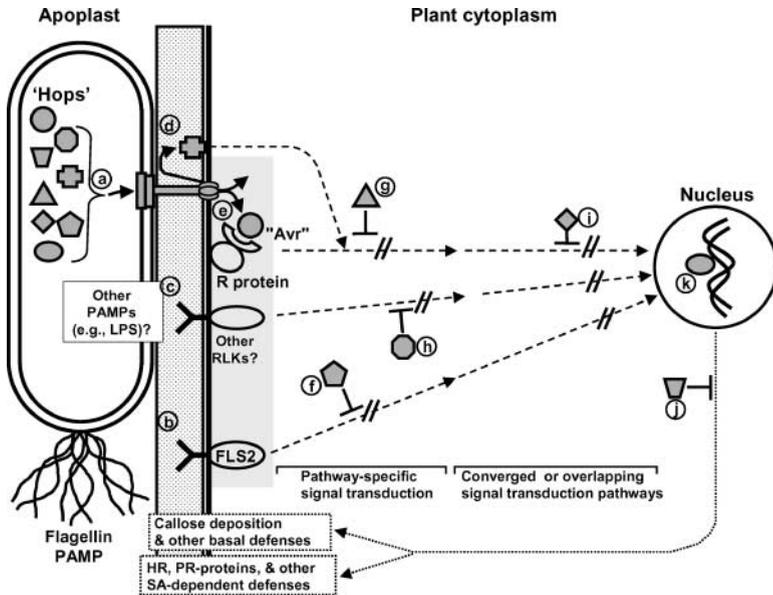


Figure 1 Model depicting sites of action for bacterial TTSS effectors as both elicitors and suppressors of plant defense. (a) Over 30 effectors may be injected into host cells by the TTSS of the model pathogen *P. syringae* pv. *tomato* DC3000. (b) *P. syringae* flagellin activates basal defense pathways following recognition by the FLS2 receptor-like kinase. (c) Other bacterial PAMPs may be recognized by additional receptor-like kinases (RLKs). (d) The TTSS-secreted harpins act in the apoplast and can trigger HR/SAR defense responses from outside of plant cells. (e) A subset of TTSS effectors (i.e., Avr proteins) are recognized inside plant cells by R proteins, which, according to the guard hypothesis, detect the activities of effectors on “guarded” susceptibility targets and activate HR defenses. The shaded box encompassing the R-protein and basal defense receptors denotes the potential relationship between guarded effector targets, the basal defense system, and the plant membrane, possibly in recognition complexes. Putative sites of action for defense-suppressive TTSS effectors include pathway-specific components for basal defenses (f and h) and HR defenses (g). Additional sites of action may be at points where signal transduction pathways converge (i). Antimicrobial responses activated by defense-signaling pathways (or involving preformed factors) also may be inhibited posttranscriptionally (j). And finally, TTSS effectors, such as AvrBs3 family members may act within the nucleus to alter host transcription (k).

As of yet, TTSS effectors that function as defense suppressors have not been described for any bacterial phytopathogens other than *P. syringae*. However, NolP from *Rhizobium* sp. NGR234 was shown recently to suppress induction of PR genes in tobacco, suggesting that one role of the TTSS in symbiotic rhizobia may be defense suppression (19). Furthermore, although the majority of animal

pathogen TTSS effectors appear to target primarily actin polymerization to alter phagocytosis, several effectors also suppress proinflammatory defense responses induced by innate immunity in animals (6, 44, 120, 164, 173). Thus, it is likely that the suppression of host defenses is a universal function of pathogen TTSSs.

Enzymatic Activities of Effectors

We have had clues to the enzymatic activities of a few Avr proteins for some time. For example, the *P. syringae* AvrD produces syringolides that elicit the HR in cultivars of soybean with race-specific resistance to races carrying *avrD* (86, 113). However, we do not know how this contributes to *P. syringae* virulence and whether AvrD can be translocated by the TTSS and produce syringolides within plant cells in addition to its known ability to produce the elicitor in bacterial cells. The *X. campestris* AvrBs2 shares similarity with *Agrobacterium tumefaciens* agrocinnopine synthase and *E. coli* glycerophosphoryl diesterase UgpQ, suggesting that AvrBs2 may make or break phosphodiester linkages in plant cells (152). Also, much has been learned about the large AvrBs3 family of effectors present in many xanthomonads (29, 96). The AvrBs3 family effectors are nuclear-localized (161, 172), can act as transcriptional activators (177, 178), and have been shown to alter transcription in plants (108). Moreover, expression of AvrBs3 family members *in planta* causes cell enlargement and hypertrophy of plant cells (52, 108).

The rapid increase in the TTSS effector inventory supports new insights into function based on recognition of motifs that predict enzymatic activities and plant targets, as recently reviewed (31, 35, 74). An emerging lesson is that many effectors appear to be cysteine proteases (Table 1). One large effector family is the YopJ/P/AvrBsT family of ubiquitin-like cysteine proteases, which is made up of proteins belonging to the CE clan of cysteine proteases, as defined by the MEROPS database (<http://merops.sanger.ac.uk/>) (120, 122). The *Yersinia* YopJ/P effector was shown to inhibit MAPK pathways and the NF κ B pathway in a manner that was dependent on the active site of the YopJ/P protease (121, 122). This results in the suppression of mammalian innate and adaptive immune systems. This family of cysteine proteases appears to be widespread in TTSS-containing phytopathogens and includes representatives in *P. syringae* (AvrPpiG1, HopPmaD, and HopPsyV) (3, 10, 48, 70), *X. campestris* (AvrRxV, AvrBst, AvrXv4, XopD, and XopJ) (73, 117, 118, 122, 169), *E. amylovora* (ORFB, Acc. No. AAF63400), *R. solanacearum* (PopP1 and PopP2) (49, 97), and likely others. These proteins are predicted to modify signal transduction pathways by removing SUMO (small ubiquitin-like modifier) modifications from components of defense pathways (122).

In an exciting recent advance, XopD was shown to act on SUMO-modified plant proteins, but not SUMO-modified animal proteins, making XopD the first protein of this class with demonstrable protease activity, although the specific plant target(s) remains unknown (73). In another exciting advance, a plant target may have been identified for the PopP2 protease. This bacterial protein was shown to interact

TABLE 1 Bacterial plant pathogen TTSS effectors: location in plant and enzymatic and defense suppressor activities

| Effector ^a | Enzymatic activity ^b | Suppression phenotype | Location ^c |
|------------------------|--|--|-----------------------|
| <i>P. syringae</i> | | | |
| AvrB | RIN4 phosphorylation (105) | ND | PM (116) |
| AvrD | Syringolide synthase (113) | ND | ND |
| AvrPphB | Cysteine protease; cleaves PBS1 (139, 140) | ND | PM (116) |
| AvrPphC | ND | Masks resident Avr activity (160) | ND |
| AvrPphE _{Pto} | ND | HopPsyA-dependent HR; PR1a expression; Bax-induced PCD in plants and yeast (79) | ND |
| AvrPpiB _{Pto} | ND | HopPsyA-dependent HR (79) | ND |
| AvrPto | ND | Callose deposition; increased growth of TTSS mutant in transgenic plants expressing AvrPto (71) | PM (138) |
| AvrPtoB (VirPphA) | ND | HopPsyA- and AvrPto-dependent HR; Bax-induced PCD in plants and yeast; H ₂ O ₂ -induced PCD in yeast (Masks resident Avr activity) (1, 76, 79) | ND |
| AvrRpm1 | RIN4 phosphorylation (105) | ND | PM (116) |
| AvrRpt2 | Cysteine protease; eliminates RIN4 (14, 15, 106) | AvrRpm1-dependent HR (131, 132); growth of TTSS mutant in transgenic plants expressing AvrRpt2 (39) | PM (15) |
| HopPtoD2 | Protein tyrosine phosphatase | Nonhost HR; MAPK-induced HR; PR1 expression; oxidative burst (26, 53) | ND |
| HopPtoE | ND | HopPsyA-dependent HR; PR1a expression; Bax-induced PCD in plants and yeast (79) | ND |
| HopPtoF (AvrPphF) | ND | HopPsyA-dependent HR; PR1a expression; Bax-induced PCD in plants and yeast (Masks resident Avr activity) (79, 160) | ND |
| HopPtoN | Cysteine protease | HopPsyA-dependent HR; reduces resident lesion formation activity in host (102) | ND |

(Continued)

TABLE 1 (Continued)

| Effector ^a | Enzymatic activity ^b | Suppression phenotype | Location ^c |
|---|---|-----------------------|-----------------------|
| <i>R. solanacearum</i> | | | |
| PopB | ND | ND | N (68) |
| PopP2 | ND | ND | N (49) |
| <i>X. campestris</i> and <i>oryzae</i> | | | |
| AvrBs3 effector family | Transcriptional activator (153, 177) | ND | N (161, 172, 177) |
| XopD | Cysteine protease (73) | ND | N (73) |
| AvrXv3 | Transcriptional activator (13) | ND ND | ND ND |

^aEffector list only includes effectors for which experimental data supports their activities and/or their location inside plant cells.

^bEnzymatic activities listed need not be direct. For example, RIN4 phosphorylation by AvrRpm1 and AvrB is not known to be direct.

^cBacterial plant pathogen effectors have been localized to either the plant plasma membrane (PM) or the nucleus (N).

ND denotes not done.

with an unusual TIR-NBS-LRR R protein, RRS-1, which contains a WRKY transcription factor domain (49). WRKY transcription factors are commonly utilized during plant defense (54). Thus, the WRKY transcription factor may be the actual virulence target, but since this factor is also involved in recognition of PopP2 in resistant plants, it is unclear whether RRS-1 is a protein targeted by the bacterium to promote disease or a plant receptor for plant defense, or both. This is one of the few examples of an NBS-LRR R protein interacting directly with an Avr protein from a pathogen and has implications for models of how R proteins recognize Avr products, as discussed below.

Another large family of TTSS effectors shared between plant and animal pathogens is the YopT/AvrPphB family of cysteine proteases, which belong to the C58 family of cysteine proteases (75, 140). Interestingly, a member of this family has not been identified in xanthomonads, whereas several have been identified in *P. syringae*, including AvrPphB (130), AvrPpiC2 (130), HopPtoC (128), and HopPtoN (102) and one in *R. solanacearum* (RSc3212) (135). YopT was shown to target the RhoA GTPase and cleave a prenyl group from the C-terminal end of RhoA, releasing it from the mammalian plasma membrane and disrupting actin polymerization, and thus preventing phagocytosis by macrophages (75, 140, 142). In an important advance, the *P. syringae* AvrPphB was shown to cleave an Arabidopsis Ser/Thr protein kinase, PBS1 (139). PBS1 is actually required for the recognition of AvrPphB on resistant plants containing the R protein RPS5 (151). Thus, the recognition of AvrPphB by the RPS5 R protein may depend on the cleavage of PBS1. In support of this notion, plants containing PBS1 mutations that prevent cleavage by AvrPphB no longer recognize AvrPphB (139).

Recently, the protein structure for AvrPphB has been determined, confirming it to be a papain-like cysteine protease (175). Another exciting finding is that the *P. syringae* effector AvrRpt2 initiates (either directly or indirectly) the elimination of the Arabidopsis RIN4 protein, which has been suggested to be a regulator of basal defenses (15, 105, 106). AvrRpt2 has a putative cysteine protease catalytic triad, which is required for elimination of RIN4 (14). The discovery of PBS1 and RIN4 as plant targets of AvrPphB and AvrRpt2, respectively, has added much to our understanding of the recognition events occurring in resistant plants, as is discussed further below.

One distinction that may be useful in categorizing TTSS effectors is whether they modify components of plant signal transduction pathways in a potentially reversible manner or simply destroy them. For example, the XopD cysteine protease noted above removes SUMO groups from plant proteins, and these presumably could be re-SUMOylated at a later point by a native plant protein. Another example of an effector that appears to reversibly modify a plant target is HopPtoD2. This *P. syringae* effector possesses protein tyrosine phosphatase (PTP) activity (26, 53). PTPs can inactivate proteins phosphorylated at tyrosine. Since plants do not have many known tyrosine kinases, the most likely targets of HopPtoD2 are MAPK pathways, and plant PTPs are likely involved in regulating these pathways. Similarly, both the *P. syringae* AvrB and AvrRpm1 effectors were shown to cause the phosphorylation of the RIN4 protein (106). However, the protein structure of AvrB recently has been determined and it does not resemble any known kinase (99). Thus, AvrB may cause RIN4 phosphorylation in an indirect manner.

Another interesting new set of effectors from *P. syringae* are HopPtoS1 (HopPtoO), HopPtoS2, and HopPtoS3 (HopPtoO2), all of which have apparent ADP ribosyltransferase (ART) active sites. The animal pathogen *P. aeruginosa* translocates ARTs via a TTSS into mammalian cells where they target a signal transduction pathway that modulates actin polymerization, thus preventing phagocytosis (63, 150). It will be interesting to compare the ART targets in plants and animals. Identifying the plant targets of all of the bacterial plant TTSS effectors will give a better understanding of plant pathogenesis, but a second likely benefit will be new tools for plant biologists exploring signaling networks.

Localization of Effectors in Plant Cells

The proper functioning of TTSS effectors is also likely to involve colocalization with targets within the host cell. Several *P. syringae* effectors (AvrB, AvrPphB, AvrPto, AvrRpm1, and AvrRpt2) have been shown to carry myristoylation signals and/or to localize to the plasma membrane (15, 116, 138). Putative myristoylation sites have been noted in other *P. syringae* effectors (e.g., HopPtoS1, HopPtoF, and AvrC) (70, 110, 128). In fact, based on their putative cleavage sites, it appears that the whole YopT/AvrPphB family of effectors in bacterial plant pathogens have putative myristoylation sites. Therefore, it is clear that the plasma membrane is

a major site of action for phytopathogen effectors. In the cases of AvrRpt2 and AvrPphB, their respective targets, RIN4 and PBS1, also are localized to the plasma membrane (15, 139).

Another destination for some phytopathogen TTSS effectors is the plant nucleus. The AvrBs3 and YopJ/P/AvrBsT families have members that localize to the nucleus (49, 73, 161, 172). The mechanisms for nuclear import may differ among effectors. For example, members of the AvrBs3 family carry nuclear localization signals (NLS) (161, 172). Pepper importin α interacted with the NLS sequences of AvrBs3 in a yeast two-hybrid analysis (153), and this host protein, along with importin β , is involved in directing eukaryotic proteins to the nucleus. In contrast, the PopP2 effector appears to help direct the R protein RRS-1 to the nucleus because RRS-1 is only nuclear-localized in the presence of PopP2 (49). Other TTSS effectors have been predicted to localize to the chloroplast of plant cells (70). Determining where other TTSS effectors localize within plant cells is another systematic method for exploring their function.

Virulence Phenotypes and Effectors as Double Agents

A subset of phytopathogen effectors is known to promote virulence in compatible interactions, and the list is likely to grow with the development of more subtle assays for mutant phenotypes (16, 90, 170). Interestingly, some effectors can promote lesion formation in susceptible hosts without a commensurate effect on bacterial growth. These include members of the AvrBs3/PthA family and HopPtoM (17, 171). HopPtoM is encoded in the conserved effector locus of *P. syringae*, which is linked to the *hrp/hrc* cluster. HopPtoN is another effector encoded in this region in *P. syringae* pv. *tomato* DC3000, which has the opposite effect on lesion formation in host tomato: a *hopPtoN* mutant produces more necrotic lesions than does wild-type DC3000 (102). But in neither the *hopPtoM* nor the *hopPtoN* mutant is there a commensurate effect on bacterial growth. Bacterial growth in planta peaks at approximately the same time and level for the two mutants and the wild-type, regardless of the severity of the lesion symptoms. One interpretation is that disease lesions represent a “delayed HR” resulting from delayed (or suppressed) recognition of HopPtoM and that suppressors like HopPtoN can further delay such defensive recognition. This scenario is consistent with the double agent abilities of many effectors and with the observed similarity in gene expression profiles of Arabidopsis plants at the time plant cells are dying in both compatible and incompatible *P. syringae* interactions (158).

Experiments with AvrPtoB in *P. syringae* pv. *tomato* DC3000 yielded a novel example of an effector acting as a double agent in promoting pathogenesis. AvrPtoB is able to suppress programmed cell death in tomato plants lacking the *Pto* resistance gene (1). However, AvrPtoB possesses an N-terminal domain that can elicit the HR in these otherwise susceptible tomato plants and a C-terminal domain that is required to suppress the HR triggered by the N-terminal domain. Thus, a DC3000 mutant that produces only the N-terminal domain of AvrPtoB is avirulent

on tomato unless complemented with full-length AvrPtoB (1). It is noteworthy that several of the effectors recently demonstrated to have HR suppressor activity are members of effector families that were previously shown to have avirulence activity (79). Thus, many effectors may be double agents, and whether their elicitor or suppressor activity prevails will depend on the complement of R proteins in the host, on the complement of effectors in the bacterium, and most likely also on quantitative factors associated with the timing and level of delivery relative to other effectors.

Effectors in the Context of Bacterium-Plant Coevolution and the Guard Hypothesis

A plausible scenario for the evolution of phytopathogen TTSS effector systems follows from considering plant basal defenses and HR defenses, the guard hypothesis (explained below), and the substantial subset of effectors now shown to have suppressor activity. The model predicts four stages in coevolution: (a) Basal defenses that are arrayed against all microbes were likely the first target of the primordial TTSS effectors (92). (b) These effectors (or more likely their activity, according to the guard hypothesis) would have come under the surveillance of the R protein system and the much stronger HR defense, which is normally triggered only by avirulent pathogens. (c) The HR defenses themselves would then become targets of a later generation of defense-suppressive effectors. (d) Continued coevolution of these pathosystems would generate populations of partners that are highly polymorphic in their respective R gene and effector gene arsenals, and outcomes would be determined by a matrix of effector-target-guard interactions involving pathogen effectors that may be both elicitors and suppressors and plant proteins that may be both susceptibility targets and defense system hair-triggers.

The guard hypothesis, which is central to this model, was proposed six years ago to explain how the prevalent class of NBS-LRR R proteins recognize Avr proteins (47, 162). Instead of the long-standing model that proposed R proteins recognize Avr proteins directly as ligands (62), the guard hypothesis proposed that R proteins protect or “guard” susceptibility factors that are targeted by pathogen effectors. Thus, R proteins detect effector-induced modifications and respond by triggering HR defenses. There are several excellent recent reviews that further explain the guard hypothesis and its limitations (24, 84, 115, 126).

Recent evidence in support of the guard hypothesis includes the finding that Avr proteins that were identified as cysteine proteases are dependent on the invariant amino acids of their active sites to be recognized by their cognate R proteins (122, 140). This suggests that each R protein is recognizing an enzymatic product of its Avr protein rather than the Avr protein directly. Also supporting the guard hypothesis are the findings discussed above that the targets for both AvrPphB and AvrRpt2 (PBS1 and RIN4) interact with their cognate R proteins, RPS5 and RPS2. That is, PBS1 interacts with the RPS5 R protein and recognition of AvrPphB by RPS5 depends on cleavage of PBS1 by AvrPphB (139). Thus, PBS1 appears to be

a virulence target of the AvrPphB cysteine protease, and RPS5 appears to closely monitor or “guard” PBS1.

Similarly, AvrRpt2 causes the elimination of RIN4, the apparent virulence target of AvrRpt2, and elimination of RIN4 triggers RPS2-mediated defenses (15, 105). In these cases, for the guard hypothesis to be correct, both RIN4 and PBS1 would need to play a role in compatible interactions in basal defense or some other process whose thwarting would benefit the pathogen. Thus, exploring the roles of PBS1 and RIN4 in defense should help determine whether the guard hypothesis is correct. It is interesting to note that a consequence of the elimination of RIN4 by AvrRpt2 is disrupted detection of AvrRpm1 and AvrB by the RPM1 R protein and suppression of the RPM1-dependent HR (15, 105). Thus, one of the strategies in pathogen coevolution in the face of guarded targets may have been the development of effectors that take out the guard. In the world of double agents, the possibilities for molecular subterfuge seem endless.

CONCLUSIONS: BRINGING DOUBLE AGENTS TO TRIAL

An important lesson from recent work with TTSS effectors is that the action of these proteins in bacterium-plant interactions is so stealthy and complex that no single experimental trial is likely to expose their multiple functions. Rather, a combination of approaches will be needed. Genomics is a powerful tool here because it enables identification of near-complete sets of effectors for given pathogens, defines effector families, and supports the identification of motifs and structural features predictive of function. Because effectors may be acting in concert and have multiple functions, assays that survey the complete collection of effectors for multiple phenotypes will be important.

We now know that the dossier for each effector in the collection should include information on existence of cognate chaperones, levels of expression and translocation during infection, subcellular localization in planta, and loss-of-function and gain-of-function assays for defense suppression activity and effects on colony initiation, growth, and symptom production. The latter tests should involve plants that are wild-type and compromised in diagnostic defense pathways. Surveys for biochemical function include assays for enzymatic activities, effector modification in planta, identification of interacting plant proteins (and modifications to them), and determination of the protein structures of effectors.

Deeper investigations of individual effectors will yield insights into effector function and, importantly, serve as foundries for the development of new assays with which to survey all effectors. Important questions to address immediately are what assays will most usefully differentiate the suppressive activities of some effectors regarding basal and HR defenses, and how are assay results influenced by great differences in the levels at which effectors are delivered in systems varying from natural infections to transformed plants with strongly expressed transgenes. For example, levels of expression may affect the localization of effectors if binding sites at the target location are limiting (15, 81). Thus, the possibility that effectors

localized to the plasma membrane may be specifically targeted to lipid rafts, which concentrate signaling molecules (95), provides one more argument for the development of assays that closely mimic infection conditions. Given the rapid progress being made in identifying and characterizing TTSS effectors, we can expect these molecular double agents to be even more important as tools for exploring the nature of plant disease and defense in the future.

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