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Cysteine proteases in phytopathogenic bacteria: identification of plant targets and activation of innate immunity

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Phytopathogenic bacteria use the type-III secretion system (TTSS) to inject effector proteins into plant cells, presumably to colonize their hosts. The function of these proteins inside plant cells has remained a mystery for years. The recent discovery that the effectors XopD, AvrXv4, AvrPphB, and AvrRpt2 have cysteine protease functions reveals that the proteolysis of host substrates is an important strategy employed by pathogens to alter plant physiology. Moreover, the characterization of these proteases and their targets provides new insight to mechanisms of bacterial virulence and the activation of plant immunity.

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Abbreviations

HR	hypersensitive response
R	resistance
RIN4	RPM1-interacting protein4
ROP	Rho-related GTPases from plants
SUMO	small ubiquitin-like modifier
TTSS	type-III secretion system
ULP1	ubiquitin-like protease1

Introduction

Phytopathogenic bacteria, including *Xanthomonas campestris*, *Pseudomonas syringae*, *Ralstonia solanacearum*, and *Erwinia amylovora*, use the type-III secretion system (TTSS) during infection to deliver bacterial proteins directly into plant cells [1]. Proteins that traverse the TTSS are commonly referred to as ‘effectors’ because they elicit disease symptoms in susceptible hosts and defense responses in resistant hosts. Numerous TTSS effectors have been identified recently, revealing that phytopathogens require multiple proteins to co-opt their hosts [2–5,6**]. TTSS effectors are known to elicit host-specific disease symptoms, to promote pathogen growth, and to suppress host defenses [7–12,13*,14,15*]. Such phenotypes suggest that some effectors function as virulence factors. At present, the

molecular basis of these effector-induced plant phenotypes is unclear. Thus, the role of TTSS effectors in bacterial colonization is still a mystery.

The plant innate immune pathway monitors bacterial infections and TTSS effectors. Disease resistance (R) proteins of the nucleotide binding site (NBS)-leucine-rich repeat (LRR) class specifically recognize TTSS effectors and then activate a localized cell death response (also known as a hypersensitive response [HR]) [16]. This defense response halts bacterial growth. The precise molecular events that mediate effector recognition in plant cells are not fully understood. To date, only a few R proteins are known to interact physically with their cognate effectors. This has prompted the hypothesis that some R proteins indirectly recognize TTSS effectors by monitoring their ‘action’ within host cells [17,18*].

In the past year, the ‘action’ of four TTSS effectors in plant cells has been elucidated. XopD [19**], AvrXv4 [20**], AvrPphB [21**,22], and AvrRpt2 [23**] are bacterial cysteine proteases that target specific plant substrates. In this review, we present a brief summary of the function of each cysteine protease family. By simply identifying effector function, we have exponentially expanded our understanding of the molecular events associated with pathogen virulence and the activation of plant disease resistance.

Pathogen-encoded SUMO proteases

YopJ and XopD effector families: protease classification

Orth and colleagues [24] discovered that the YopJ effector family shares structural similarity with cysteine proteases. This pivotal observation inspired others to search for structural similarity for other TTSS effectors. Since then, both YopJ and XopD-like effectors [19**] have been assigned to the CE clan of cysteine proteases (Table 1). Proteases in this clan are characterized by a cysteine nucleophile and a catalytic core that is comprised of three amino-acid residues, H/E/C or H/D/C. Within the CE clan, the YopJ family has been assigned to the C55 peptidase family and the XopD family to the C48 peptidase family (see MEROPS Protease Database for details [<http://merops.sanger.ac.uk/>]). The targeted disruption of the predicted catalytic core of YopJ from *Yersinia* [24], and of AvrBsT [24], AvrXv4 [20**], and XopD [19**] from *Xanthomonas*, abolishes the phenotype of each protein in their respective host, supporting the hypothesis that these families encode cysteine proteases.

Orth and colleagues [24] also noted that the YopJ family shares limited structural similarity with the yeast

Table 1

Pathogen TTSS cysteine protease effectors and their presence in bacteria that are associated with plants.

(a) Pathogen TTSS cysteine protease effectors

TTSS family	Named after	Protease assignment	Distribution in bacteria
YopJ family	<i>Yersinia</i> sp. YopJ	CE clan, C55 peptidase	Wide, animal and plant associated
XopD family	<i>Xanthomonas campestris</i> XopD	CE clan, C48 peptidase	Plant associated
YopT family	<i>Yersinia</i> sp. YopT	CA clan, C58 peptidase	Wide, animal and plant associated
AvrRpt2 family	<i>P. syringae</i> AvrRpt2	CA clan, C47 peptidase	Unique, plant associated

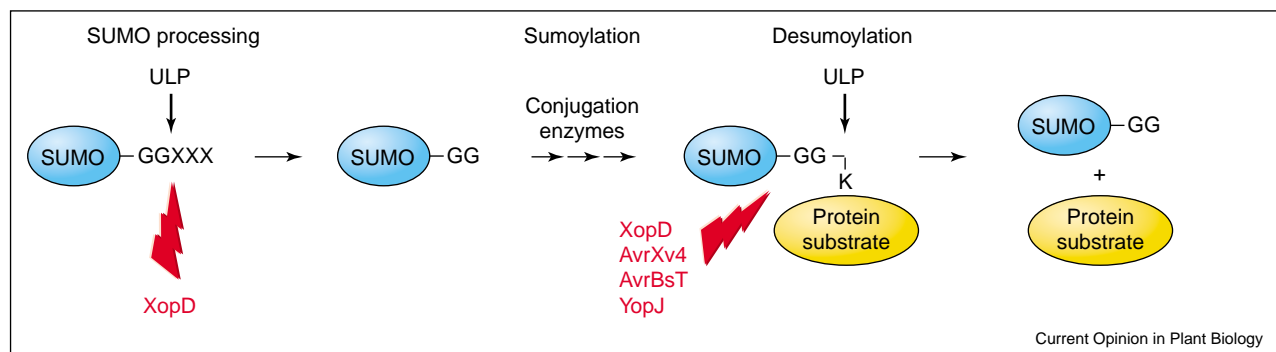
(b) Presence of cysteine protease effectors in bacteria associated with plants

TTSS family	Bacterium	Protein designation	Plant substrate
YopJ family	<i>Xanthomonas campestris</i>	AvrXv4, AvrBsT, AvrRxv, XopJ	SUMO–protein conjugates
	<i>Ralstonia solanacearum</i>	PopP1, PopP2, PopP3	
	<i>P. syringae</i> pv. <i>pisii</i>	AvrPpiG1	
	<i>P. syringae</i> pv. <i>maculicola</i>	HopPmaD	
	<i>Erwinia pyrifoliae</i>	ORF B	
	<i>Rhizobium</i> sp. NGR234	Y4IO	
XopD family	<i>Xanthomonas campestris</i>	XopD	SUMO–protein conjugates
	<i>P. syringae</i> pv. <i>eriobotryae</i>	PsvA	
YopT family	<i>P. syringae</i> pv. <i>phaseolicola</i>	AvrPphB	PBS1
	<i>P. syringae</i> pv. <i>pisii</i>	AvrPpic2	
	<i>P. syringae</i> pv. <i>tomato</i>	HopPtoC, HopPtoN	
	<i>Bradyrhizobium japonicum</i>	BLR2140, BLR2058	
	<i>Rhizobium</i> sp. NGR234	Y4zC	
AvrRpt2 family	<i>P. syringae</i> pv. <i>tomato</i>	AvrRpt2	RIN4

ubiquitin-like Protease1 (ULP1), a cysteine protease in the C48 family. This led them to propose that these effectors may mimic the activity of ULPs (also known as small ubiquitin-like modifier [SUMO] proteases) inside eukaryotic hosts [25]. ULPs dynamically and reversibly regulate the SUMO modification pathway (Figure 1). SUMO is posttranslationally linked to proteins by a conjugation system that is mechanistically analogous to the ubiquitin system [26,27^{••}]. SUMO modification or ‘sumoylation’ controls several cellular processes [26,28^{••}],

including nuclear transport, enzyme activity, transcriptional activation and repression, and cell-cycle progression. In plants, sumoylation is linked to pathogen attack [29], abiotic stress [27^{••}], hormone signaling [30[•]], and flowering time [31[•]]. The large number of ULPs in *Arabidopsis* [27^{••},31[•]] suggests that ‘desumoylation’ may be a critical regulatory step controlling protein function. A pathogen that employs effectors to mimic ULPs could potentially disrupt many cellular processes, giving the pathogen a selective advantage over its host.

Figure 1



Schematic representation of the SUMO pathway in eukaryotes. SUMO, the small ubiquitin-like modifier, is posttranslationally linked to proteins by a conjugation system that is mechanistically analogous to the ubiquitin system. SUMO modification (‘sumoylation’) is a reversible process that is controlled by ULPs. As peptidases, ULPs cleave an inactive SUMO precursor to generate a mature modifier that has an exposed carboxy-terminal glycine residue (G). This glycine residue forms an isopeptide bond between the carboxyl terminus of SUMO and the epsilon amino group of a lysine residue (K) of a protein substrate. As isopeptidases, ULPs cleave SUMO (‘desumoylation’) from the SUMO–protein conjugate. The *Xanthomonas* XopD, AvrXv4 and AvrBsT effectors, and the *Yersinia* YopJ effector, mimic ULP enzymatic activity, disrupting the regulation of the SUMO pathway.

As predicted, the transient expression of YopJ [24] in animal cells and of AvrBsT (A Hotson, MB Mudgett, unpublished) and AvrXv4 [20**] in plant cells reduces the level of SUMO–protein conjugates, indicating that these three effectors possess SUMO–isopeptidase activity *in vivo*. Despite much effort, however, constitutive enzyme activity has not been detected for these effectors *in vitro*. This indicates that YopJ, AvrBsT and AvrXv4 probably require host factors for activation. Together, these studies indirectly support the hypothesis that YopJ-like effectors function as SUMO proteases within host cells.

Direct proof of this hypothesis came from studies of the *Xanthomonas* XopD effector [19**,32]. Biochemical studies demonstrated that XopD is a constitutively active cysteine protease that possesses both SUMO peptidase and isopeptidase activity [19**]. Interestingly, purified XopD cleaves only a tomato SUMO precursor and fails to recognize a similar mammalian substrate. This shows that XopD substrates are host-specific. The substrates for XopD are expected to be in the plant nucleus because XopD is localized to subnuclear foci [19**]. Overall, these studies confirm that XopD mimics plant SUMO proteases.

Virulence role of pathogen SUMO proteases?

Several proteins are known to be sumoylated in plants [19**,30*,31*] but their identities have not yet been revealed. Thus, we are naïve with regard to the physiological consequence of effector-mediated SUMO proteolysis during plant infection. Given that SUMO is particularly important for transcriptional regulation, we speculate that XopD may target transcription factors at subnuclear foci [28**]. The redundancy of SUMO proteases in *Xanthomonas* may explain why XopD and AvrXv4 do not appear to play a crucial role in pathogen virulence [20**,32].

Plant defense against pathogen SUMO proteases

Resistance to the *Ralstonia* YopJ-like effector, PopP2 [33*], provides insight into the mechanisms that plants use to combat pathogen SUMO proteases. The *Arabidopsis* R protein RRS1 [34] interacts with PopP2 in a yeast two-hybrid assay, suggesting that RRS1's molecular recognition of PopP2 is direct [33*]. Within the plant cell, cytoplasmic RRS1 moves to the plant nucleus in the presence of PopP2 [33*]. This is intriguing considering that RRS1 encodes an atypical Toll interleukin-repeat (TIR)-NBS-LRR protein that has a WRKY domain, a signature feature of plant transcription factors. Hence, RRS1 activation and trafficking to the nucleus may directly initiate the transcription of defense genes. Determining whether PopP2 is a SUMO protease will reveal the events that are required for its recognition and interaction with RRS1. The recognition of other YopJ-like effectors (AvrXv4 and AvrBsT) appears to be indirect as mutants in which the catalytic cores of

these effectors are disrupted do not elicit a HR in resistant plants [20**,24].

The YopT cysteine protease family

YopT family: protease classification

The YopT family shares secondary structure with cysteine proteases ([22]; Table 1). These effectors belong to the C58 family in the CA clan. At present, this family has 19 known members, all from pathogenic and symbiotic bacteria. All members of the YopT family possess the invariant C/H/D catalytic core. The crystal structure of AvrPphB from *P. syringae* shows that AvrPphB's core closely resembles that of papain-like cysteine proteases in the CA clan, confirming the protease assignment of this effector [35**]. Interestingly, AvrPphB residues that define substrate binding are divergent among family members. This predicts that family members will probably have different substrate specificities and thus target different host proteins. The demonstration that AvrPphB and YopT possess enzymatic activities that cleave distinct host substrates supports this hypothesis [21**,22].

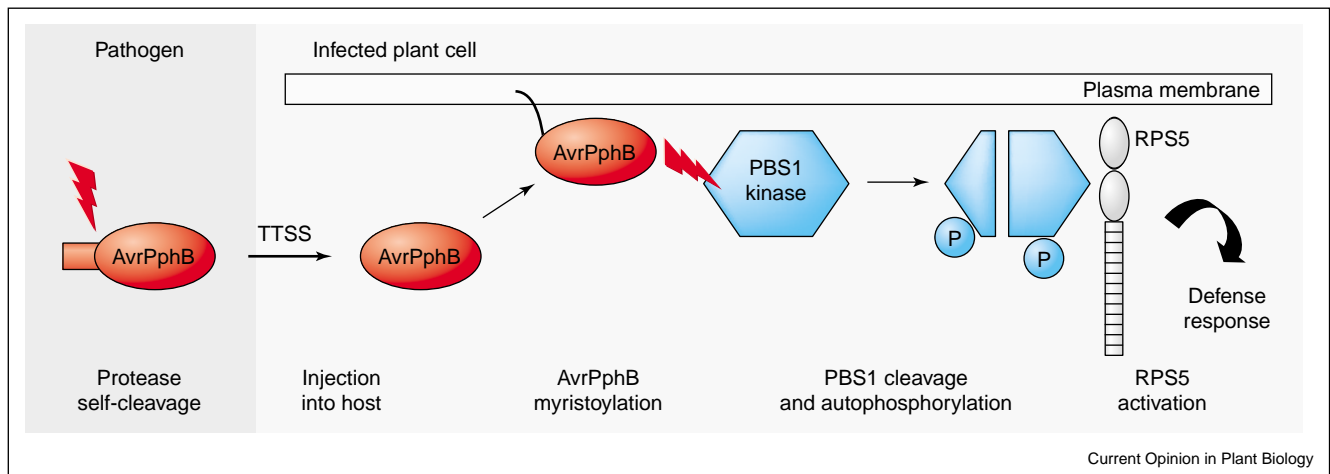
The *P. syringae* AvrPphB effector

The discovery that AvrPphB encodes a protease explains how AvrPphB is cleaved in the pathogen, and how it activates defense responses in plants (Figure 2). AvrPphB is a 35-kDa polypeptide that is amino-terminally cleaved within the pathogen to form a 7-kDa and a 28-kDa product [36]. AvrPphB cleavage exposes a fortuitous myristoylation sequence at the amino-terminus of the 28-kDa product, suggesting that it is modified in the plant cell and then trafficked to the plasma membrane. Nimchuk and colleagues [37] showed that AvrPphB is fatty acylated and associated with plant membrane fractions, supporting a role for AvrPphB at the host plasma membrane.

AvrPphB triggers a HR in the cells of *Arabidopsis* plants that express the RPS5 protein [38]. The activation of RPS5 requires a second *Arabidopsis* protein, the PBS1 serine/threonine kinase [39]. The function of PBS1 is not yet known; PBS1 displays autophosphorylation activity, however, confirming that it is a functional kinase [39]. Curiously, putative myristoylation motifs are also present in RPS5 and PBS1, suggesting that AvrPphB–RPS5–PBS1 physical interactions that lead to the activation of RPS5 may occur at the plasma membrane. Consistent with the prediction that AvrPphB encodes a cysteine protease, AvrPphB is not cleaved and does not induce an RPS5-dependent HR if its catalytic core is mutated [22]. This clearly demonstrates that the cleavage of AvrPphB is autocatalytic. Moreover, this indicates that AvrPphB-dependent proteolysis of a host substrate is required for the activation of RPS5.

Shao and colleagues [21**] showed that PBS1, and not RPS5, is the plant substrate for AvrPphB. PBS1 physically

Figure 2



Model of AvrPphB protease action in resistant RPS5 *Arabidopsis* plants that are infected with *P. syringae*. AvrPphB cleaves itself before TTSS delivery into plant cells. *In planta*, AvrPphB is fatty acylated and targeted to the plasma membrane. PBS1 is then cleaved by AvrPphB. PBS1 cleavage and auto-phosphorylation are both required for the activation of RPS5-mediated transduction of defense signals.

interacts with AvrPphB *in planta* and, as a result, is cleaved into two polypeptides. *In vitro*, purified recombinant AvrPphB cleaves itself as well as purified recombinant PBS1 [21^{••}]. This demonstrates that AvrPphB is a constitutively active enzyme that requires no host factors for PBS1 proteolysis. AvrPphB cleaves PBS1 after the sequence 'GDK', a motif that defines AvrPphB substrate specificity for self-proteolysis. Mutation of the PBS1 'GDK' motif abolishes AvrPphB-dependent cleavage of PBS1 [21^{••}]. Interestingly, both PBS1 cleavage and PBS1 kinase activity are required for RPS5 resistance [21^{••}]. Taken together, these studies suggest that the association of AvrPphB with PBS1 at the host plasma membrane releases a PBS1 fragment that is possibly phosphorylated. This fragment then acts as the molecular trigger to activate RPS5 signal transduction.

Virulence role of *Yersinia* YopT effector

AvrPphB protease activity in part explains the activation of plant defenses, yet provides little insight to the role of this effector in pathogenesis. Conversely, the activity of the *Yersinia* YopT protease provides valuable insight into its cytotoxic effect in mammalian cells. YopT is cytotoxic because it disrupts the actin cytoskeleton upon entry into the cell, resulting in severe membrane deformation. Such cellular rearrangement occurs because YopT interacts with lipid-modified Rho GTPases, which are key players in actin assembly [22]. YopT cleaves the prenyl moiety from Rho GTPases, releasing them from the plasma membrane [22]. As actin formation is crucial for the phagocytosis and gene transcription that are associated with host immunity, YopT action may also interfere with other host pathways during pathogenesis.

A virulence role for AvrPphB?

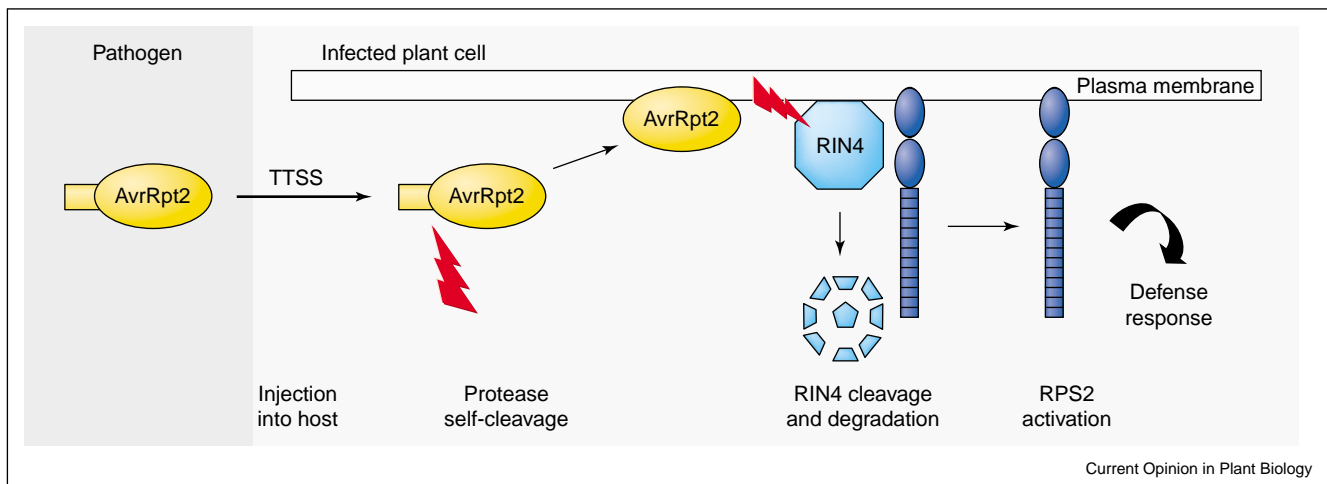
Work done on the YopT protease may provide clues as to the virulence role of AvrPphB in plant cells. Plants lack Rho GTPases but have maintained a subfamily of the Rho family, known as the Rho-related GTPases from plants (ROPs) [40[•]]. Interestingly, ROPs have been linked to plant defense responses. In rice, the ROP OsRac1 initiates the production of reactive oxygen species that is essential for resistance to the rice blast fungus [41]. It is interesting to speculate that, in addition to cleaving PBS1, AvrPphB may interact with and cleave small GTPases. The disruption of small GTPases in plants could provide the pathogen with a direct mechanism for the suppression of host defenses. Identifying the function of PBS1, and possibly new AvrPphB substrates, will hopefully give us more clues to AvrPphB's role in *P. syringae* pathogenesis.

The AvrRpt2 cysteine protease

AvrRpt2 family: protease classification

The *P. syringae* AvrRpt2 protein is the latest TTSS effector shown to resemble a cysteine protease ([23^{••}]; Table 1). To date, no AvrRpt2 homologs have been found in the sequenced genomes of bacterial pathogens, revealing that this effector is unique. Axtell and colleagues [23^{••}] showed that AvrRpt2 shares structural similarity with the catalytic core of staphopain, a cysteine protease in the C47 family within the CA clan. This was a key breakthrough because AvrRpt2 protease activity could help to explain two phenotypes that are associated with this effector: first, AvrRpt2-dependent activation of the RPS2 R protein, and second, AvrRpt2's interference with the RPM1 R protein.

Figure 3



Model of AvrRpt2 protease action in resistant RPS2 *Arabidopsis* plants that are infected with *P. syringae*. AvrRpt2 undergoes autocatalysis *in planta*. Membrane-associated AvrRpt2 then cleaves RIN4, leading to RIN4 degradation and the activation of RPS2-mediated defense signal transduction.

AvrRpt2 activation of RPS2

Although a direct enzymatic activity has not yet been demonstrated for AvrRpt2, a wealth of genetic and molecular data strongly suggest that AvrRpt2 functions as a cysteine protease *in planta* (Figure 3). *P. syringae* translocates the mature 28-kDa AvrRpt2 protein into plant cells, where it is amino-terminally processed to 7-kDa and 21-kDa products [42]. The carboxy-terminal 21-kDa product is sufficient to trigger a defense response in *Arabidopsis* plants that express the RPS2 protein [42]. More importantly, AvrRpt2 action in *Arabidopsis* causes an RPS2-independent disappearance of the novel host protein RPM1-interacting protein4 (RIN4) [43^{••},44,45^{••}]. This prompted Axtell and colleagues [23^{••}] to explore the possibility that AvrRpt2 encodes a RIN4 protease. They discovered that AvrRpt2 possesses a protease catalytic triad C/H/D. Indeed, mutation of the catalytic core prevents AvrRpt2 proteolysis, RIN4 degradation, and the activation of RPS2-mediated resistance [23^{••}]. This suggests that the cleavage of AvrRpt2 is mediated through an autocatalytic event. Furthermore, this implies that RIN4 is a direct substrate for AvrRpt2. Efforts to show that purified recombinant RIN4 is cleaved by purified recombinant AvrRpt2 have been unsuccessful. This is not surprising considering that AvrRpt2 processing requires a eukaryotic factor [42,46[•]]. Identifying this elusive host factor will reveal the mechanism of AvrRpt2 activation and substrate cleavage.

The fact that direct interactions between AvrRpt2 and RPS2 have not been observed suggests that the molecular recognition of AvrRpt2 is indirect. Interestingly, AvrRpt2, RPS2, RIN4, and RPM1 are each associated with the host plasma membrane [44,45^{••},47], and RIN4 physically

interacts with RPS2 [45^{••}] and RPM1 [44]. This predicts that all four proteins may reside in close proximity, possibly in a protein complex, during plant infection. AvrRpt2-dependent cleavage of RIN4 may alter such a complex, triggering the specific activation of RPS2 and, thus, the indirect recognition of AvrRpt2.

AvrRpt2 virulence and interference with RPM1

Transgenic *rps2 Arabidopsis* plants that express AvrRpt2 display an enhanced disease susceptibility phenotype when infected with *P. syringae* [11]. This clearly shows that AvrRpt2 has a virulence role *in planta*. AvrRpt2 interference with RPM1 resistance [48,49] suggests that AvrRpt2's virulence role may include the elimination of RIN4. Physical interactions of AvrRpt2, RIN4, and RPM1 at the plasma membrane could explain the mechanism of AvrRpt2 interference. In the absence of AvrRpt2, the *P. syringae* effectors AvrRpm1 and AvrB elicit defense responses in plants that express RIN4 and RPM1 [44]. RIN4 is required for the proper localization and accumulation of RPM1 at the plasma membrane [44]. AvrRpm1 and AvrB independently interact with RIN4 and induce its phosphorylation [44]. This stimulates RPM1 activation. In the presence of AvrRpt2, AvrRpm1 and AvrB do not activate RPM1 because RIN4 is degraded [43^{••},45^{••}]. These studies show that *P. syringae* uses three different effectors to modify or eliminate RIN4. What then is the role of RIN4? And, why do plant cells use two different R proteins to monitor RIN4?

Conclusions

These studies have opened our eyes to the strategies used by plant pathogenic bacteria to infect plant cells. We now know that the proteolysis of host proteins is a conserved

mechanism used by diverse pathogens to manipulate host signal transduction. Furthermore, we now have direct evidence that phytopathogenic TTSS effectors encode functional cysteine proteases, proteolyze specific host targets, and are indirectly recognized by R proteins as a consequence of their action in plant cells. The challenge now is to identify more host substrates and to elucidate their function in plant physiology. This will be essential if we are ever going to understand how the manipulation of host proteins allows pathogens to grow and persist in plants.

Acknowledgements

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