Deciphering the mode of action and host recognition of bacterial type III effectors

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Abstract. Plant pathogenic bacteria adhere to cell walls and remain external to the cell throughout the pathogenic lifecycle, where they elicit host immunity through host plasma membrane localised receptors. To be successful pathogens, bacteria must suppress these defence responses, which they do by secreting a suite of virulence effector molecules into the host cytoplasm. However, effectors themselves can act as elicitors after perception by intracellular host immune receptors, thus, re-activating plant immunity. Bacterial effectors generally target host molecules through specific molecular activities to defeat plant defence responses. Although effectors can be used as tools to elucidate components of plant immunity, only a handful of these molecular targets are known and much remains to be learnt about effector strategies for bacterial pathogenicity. This review highlights recent advances in our understanding of the mode of action of bacterial effectors, which in the future will lead to improvements in agriculture.

Additional keywords: bacteria, defense, effector, NB-LRR, plant immunity, PRR.

Introduction

Gram-negative bacteria of the genera *Pseudomonas*, *Xanthomonas*, *Ralstonia*, *Pantoea* and *Erwinia* are extracellular pathogens that use a specialised type-III secretion apparatus (TTSS) to deliver virulence effector proteins into the plant cell (Jones and Dangl 2006). Collectively, these effectors manipulate host cells to optimise the pathogenic niche, particularly through suppression of the plant immune system (Jones and Dangl 2006). Plants have two sensory systems to detect pathogens; the first branch is based on extracellular recognition of highly conserved elicitors called pathogen-associated molecular patterns (PAMPs) by transmembrane pattern recognition receptors (PRRs) that typically encode receptor kinases (RKs) (Jones and Dangl 2006). Perception of PAMPs by PRRs activates PAMP-triggered immunity (PTI) and prevents further colonisation of the host (Jones and Dangl 2006). The best described examples in *Arabidopsis* include the recognition of the bacterial PAMPs flagellin and elongation factor-Tu (EF-Tu) by the RKs FLAGELLIN SENSING 2 (FLS2) and EF-TU RECEPTOR (EFR), respectively (Gómez-Gómez and Boller 2000; Zipfel *et al.* 2006). The second sensory node operates within the cytoplasm and is triggered by specific intracellular recognition of certain effector proteins by host nucleotide binding-leucine rich repeats (NB-LRR) proteins. Activation of NB-LRR proteins results in effector-triggered immunity (ETI) and is normally accompanied by a

hypersensitive cell death response (HR) at the infection site (Jones and Dangl 2006). Extracellular recognition by PRRs is generally conserved across broad taxonomic boundaries (Boller and Felix 2009), whereas intracellular recognition by NB-LRRs is typically polymorphic even within plant species (Dangl and Jones 2001). Bacterial effectors are known to suppress immune signalling events emanating from both PRRs and NB-LRRs (Nomura *et al.* 2006; Fu *et al.* 2007; Rosebrock *et al.* 2007; Zhang *et al.* 2007; Göhre and Robatzek 2008; Shan *et al.* 2008; Gimenez-Ibanez *et al.* 2009; Guo *et al.* 2009). However, the function and molecular host targets of most effectors remain to be elucidated. Identification of such targets is currently an important area of research in plant–pathogen interactions. The battle between plants and disease-causing pathogens has been compared with an arms race, with each innovation in host defence requiring a corresponding change in infection strategy from the pathogen, although neither entity gains the upper hand for long. This review explores emerging concepts of effector action and identification of host targets within the plant cell.

The effector sets of plant pathogenic bacteria: unique combinations with highly redundant functions

Genome sequencing has enabled annotation of the effector repertoires of five *Pseudomonas syringae* (*P. syringae*) strains, including *Pto* DC3000, *Pto* T1, *P. syringae* pv. *syringae* (*Pss*) B728a, *P. syringae* pv. *phaseolicola* (*Pph*) 1448A, and

P. syringae pv. *tabaci* (Pta) 11528 (Buell *et al.* 2003; Feil *et al.* 2005; Joardar *et al.* 2005; Almeida *et al.* 2009). These possess both overlapping and distinct host ranges and represent the three major Pseudomonad pathovar clades (Sawada *et al.* 1999). Individual bacterial strains possess 20–30 effectors. Unexpectedly, the effector repertoires of these strains are strikingly different, even for strains such as *Pto* and *Pss*, which infect the same host (Joardar *et al.* 2005; Studholme *et al.* 2009). Thus, the composition of each effector set has so far failed to explain the host specificity of different bacterial strains, except where host recognition of effectors limits infectivity, as exemplified by the *Pto* DC3000 effector HopQ1–1 (Wei *et al.* 2007). Another general feature of effector sets is that they are collectively essential for pathogenesis but individually dispensable (Kvitko *et al.* 2009). Mutations that affect individual effector genes typically cause no or very subtle reductions in bacterial virulence. The reason underlying these observations is that effectors work redundantly – different effectors target the same host molecules through distinct strategies. This has been demonstrated both genetically and molecularly. Almost all effectors encoded by *Pto* DC3000 have the ability to suppress immune responses in at least one assay (Guo *et al.* 2009). Why do bacterial pathogens need such large effector repertoires with overlapping functions? First, as demonstrated experimentally, it means that individual effectors are dispensable. This allows effector loss without compromising infectivity. This may be important, for example, to allow the pathogen to discard a specific effector that is recognised by a plant NB-LRR protein without affecting its virulence, which provides an explanation for why NB-LRR mediated disease resistance in crops is often transient. Redundancy would allow an existing effector to evolve a novel or enhanced virulence function on a new host, because the original virulence activity would be retained by the redundant partner. Finally, it is possible that not all effectors are active on all plant species, so a level of redundancy is required to retain certain virulence functions on all hosts. This inbuilt redundancy makes functional analysis of effectors difficult. Thus, combinatorial effector gene deletions, or individual delivery of effectors using various experimental tools, are useful strategies to remove redundancies and identify effectors that make key contributions to disease *in planta* (Kvitko *et al.* 2009).

Molecular activities of bacterial effectors

Several detailed studies showing targeting of host molecules by bacterial type-III effectors have been published. This section summarises the most recent examples.

Suppression of PAMP-triggered immunity (PTI)

Bacteria are betrayed by PAMPs such as flagellin or EF-Tu during the early stages of infection. These molecules are sensed by PRRs, many of which rapidly form a heterocomplex with a second RK called BRI1-ASSOCIATED KINASE 1 (BAK1) shortly after elicitation (Chinchilla *et al.* 2007; Heese *et al.* 2007). Two unrelated *Pto* DC3000 effectors, AvrPto and AvrPtoB, interact with and inhibit the signalling of several RKs involved in PTI, including FLS2, EFR, and BAK1 (Gohre *et al.* 2008; Shan *et al.*

2008; Xiang *et al.* 2008; Gimenez-Ibanez *et al.* 2009). Different models state that AvrPto acts by directly binding and inhibiting the kinase activity of PRRs (Xiang *et al.* 2008), or by abrogating the formation of the FLS2-BAK1 complex required for signalling (Shan *et al.* 2008). These models are not mutually exclusive and remain to be resolved. In contrast to AvrPto, AvrPtoB is a multidomain protein containing a C-terminal E3 ubiquitin ligase domain that tags substrate proteins for subsequent degradation (Rosebrock *et al.* 2007; Gohre *et al.* 2008; Gimenez-Ibanez *et al.* 2009). Like AvrPto, AvrPtoB disrupted the FLS2-BAK1 complex (Shan *et al.* 2008), and its N-terminus seems to comprise a protein kinase binding domain that specifies interaction with at least five intracellular substrates (Wu *et al.* 2004; Rosebrock *et al.* 2007; Gohre *et al.* 2008; Shan *et al.* 2008; Gimenez-Ibanez *et al.* 2009). In addition, its E3 ligase domain is required for degradation of the PRR CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) (Gimenez-Ibanez *et al.* 2009) and partially degrades FLS2 (Gohre *et al.* 2008; Gimenez-Ibanez *et al.* 2009). Overall, it seems to use a bipartite strategy for suppression of kinase activity, with a variable requirement for the C-terminal ubiquitin ligase activity. Because both AvrPto and AvrPtoB target a commonly-used fold in plants – the RK/Pelle class of protein classes (Shiu and Bleecker 2003) – they may have many more cellular targets.

A further illustrative example concerns proteolytic cleavage of the *Arabidopsis* cytoplasmic kinase PBS1 by the *P. syringae* effector AvrPphB (Shao *et al.* 2003). The pathogenic strategy underlying this event was previously unclear because mutant *pbs1 Arabidopsis* plants are not more susceptible to bacterial infections than wild-type plants. Recently though, Zhang and colleagues have shown that in addition to cleavage of PBS1, AvrPphB targets 10 PBS1-like (PBL) kinases, including BIK1 (BOTRYTIS-INDUCED KINASE 1), PBL1, PBL2, and PBS1 (Zhang *et al.* 2010). In unstimulated plants, BIK1 and PBL1 interact with FLS2 and are rapidly phosphorylated after activation of FLS2. The ligand-dependent release of BIK1 from FLS2 is in striking contrast to the previously described ligand-dependent complex formation between FLS2 and BAK1 (Chinchilla *et al.* 2007; Heese *et al.* 2007). However, the role of BIK1 in PTI is unclear because *Arabidopsis* mutants lacking this gene show constitutive activation of defences. Nevertheless, AvrPphB apparently inhibits PTI by cleaving PBL kinases. BIK1 and PBL1 probably integrate signalling from multiple immune receptors into downstream responses as their functions seem to be additive (Zhang *et al.* 2010). These results raise the question of whether AvrPphB targets only those PBL kinases with roles in immunity, or lacks specificity for its substrates.

Additional strategies are used by bacterial effectors to suppress PTI downstream of PRR activation. For example, the *Pto* DC3000 effector HopAII suppresses mitogen-activated protein kinase (MAPK) activation via its phosphothreonine lyase activity (Zhang *et al.* 2007). MAPKs pathways transfer information from receptors to cellular responses and operate downstream of FLS2 and likely other PRRs (Asai *et al.* 2002; Suarez-Rodriguez *et al.* 2007). Despite this, He *et al.* (2006) previously observed neither MAPK suppression nor reductions in defence gene expression in the presence of HopAII. Thus, the precise role of this effector in pathogenicity remains to be defined.

Microbial perception by extra- and intra-cellular receptors orchestrates extensive reprogramming of gene transcription in favour of defence. The effector HopU1, which encodes a mono-ADP-ribosyltransferase (ADP-RT), suppresses plant innate immunity by modifying glycine-rich RNA-binding proteins (GR-RBPs) that possess RNA-recognition motifs (RRMs) such as GRP7 (Fu *et al.* 2007). Consistently, *Arabidopsis* plants lacking the *grp7* gene are more susceptible to *Pto* DC3000 infection. The ADP-RT catalytic residues are required for the known defence-suppression activities of HopU1 (Fu *et al.* 2007). GRP7 has characteristics of an RNA binding protein, but its role(s) in plant immunity is unknown. The ADP-ribosylation of GRP7 by HopU1 may interfere with the ability of GRP7 to bind RNA. This mechanism could represent a strategy to alter RNA stability and, thus, modify the expression of a significant proportion of the plant defence transcriptome. Further work is required to discover more about the *in vivo* functions of the RNA-binding proteins that are ADP-ribosylated by HopU1 and their roles in plant innate immunity.

Antimicrobial compounds are thought to be secreted by exocytosis (Kalde *et al.* 2007). To prevent this secretion, effectors can act on proteins involved in vesicle trafficking and modify secretion of such compounds. Two effectors, HopM1 of *Pto* DC3000 and XopJ of *Xanthomonas campestris* pv. *vesicatoria*, interfere with this pathway (Fig. 1) (Nomura *et al.*

2006; Bartetzko *et al.* 2009). How XopJ alters vesicle trafficking is not known. In contrast, detailed analyses reveal that HopM1 exploits the plant ubiquitination system to modify vesicle trafficking. HopM1 mediates the destruction of an immunity-associated adenosine diphosphate (ADP) ribosylation factor – guanine nucleotide exchange (ARF-GEF) factor named MIN7 (*Arabidopsis thaliana* (L.) Heynh HopM interactor 7) via the host proteasome (Nomura *et al.* 2006). ARF-GEFs are well known components of vesicle trafficking (Steinmann *et al.* 1999). *AtMIN7* knockout mutants do not deposit callose into cell walls in response to flagellin treatment, and allow increased growth of the TTSS-deficient strain *Pto* DC3000 *hrcC* (Nomura *et al.* 2006). The MIN7 protein may be important for the accumulation of many defence-induced antimicrobial compounds at the infected site, but it is conceivable that it plays multiple roles in plant immunity, for example, biogenesis of PRRs.

It remains an open question as to why a virulent bacterium such as *Pto* DC3000 grows more on *Arabidopsis* plants lacking FLS2 when it has several effectors that are able to suppress this pathway. One possibility is that the timing of PTI elicitation relative to its effector-mediated suppression is relevant. PTI must be activated immediately upon invasion of the leaf by bacteria. In contrast, bacteria need to assemble the TTSS apparatus before secreting the effectors into the plant cell, a process that must take some hours. Thus, effectors presumably act to slow or stop active PTI

Fig. 1. Schematic diagram illustrating the various steps at which bacterial type III effectors interfere with plant innate immunity.

signalling that has already been established. Such sequential events were previously postulated in the ‘zigzag’ model (Jones and Dangl 2006).

Entry sites for bacterial invasion: hijacking stomata?

Following proliferation on the leaf surface, bacteria use natural openings such as stomata to enter the plant tissue. Stomatal guard cells express PRRs that sense bacteria and induce stomatal closure, limiting bacterial access to the leaf (Melotto *et al.* 2006). To re-open stomata, *Pseudomonas syringae* pv. *tomato* (Pto) DC3000 produces the phytotoxin coronatine, a mimic of the phytohormone jasmonic acid (JA) (Melotto *et al.* 2006, 2008). However, not all *Pseudomonas* strains produce coronatine, raising the question of which alternative strategies are used to open stomata. Recent reports indicate that *Arabidopsis* RPM1-INTERACTING PROTEIN 4 (RIN4) functions with the plasma membrane H⁺-ATPases AHA1 and AHA2 to regulate stomatal aperture (Liu *et al.* 2009). RIN4 is a key regulator of plant immunity, and is targeted by the type-III effectors AvrB, AvrRPM1 and AvrRpt2 (Mackey *et al.* 2002, 2003; Axtell and Staskawicz 2003). RIN4 is expressed in guard cells, and *RIN4* overexpression and knockout lines show enhanced and reduced H⁺-ATPase activity respectively (Liu *et al.* 2009). The *rin4* knockout line exhibits constitutive stomatal closure and congruently, enhanced bacterial resistance, likely by inhibiting bacterial entry to the leaf (Liu *et al.* 2009). It is difficult to reconcile this model with the observations that at least three *Pseudomonas* type-III effectors target RIN4 for destruction or modification, with the predicted outcome that this would limit virulence through stomatal closure. Recently, it was suggested that a fourth effector, HopF2, also targets RIN4, with implications for virulence (Wilton *et al.* 2010). It is still unclear why so many bacterial effectors target RIN4 and whether any bacterial effector truly functions in reopening stomata; one recently reported possibility is that AvrB activates mitogen activated protein kinase 4 (MPK4) via RIN4, to perturb hormone signalling and promote bacterial virulence (Cui *et al.* 2010). However, it has not been unequivocally demonstrated that targeting of RIN4 by effectors enhances bacterial virulence. Moreover, RIN4 targeting by the effectors AvrB, AvrRpm1 and AvrRpt2 causes activation of ETI in presence of the NB-LRRs RPS2 or RPM1, which form constitutive protein complexes with RIN4 (Mackey *et al.* 2002, 2003; Axtell and Staskawicz 2003). Further work could to determine whether these effectors evolved in the first instance to manipulate stomatal aperture by targeting RIN4, and whether that explains the subsequent incorporation of RIN4 into immune recognition complexes as a specificity determinant.

Direct manipulation of host gene expression by TAL effectors

The transcription activator-like (TAL) effectors of *Xanthomonas* spp. are important virulence factors that act in the host nucleus to manipulate gene expression (Boch *et al.* 2009). TAL effectors induce cell host hypertrophy and modulate disease symptomatology (White *et al.* 2009). A recent milestone discovery deciphered how the DNA-binding specificity of TAL effectors is encoded. All TAL effectors contain highly

conserved N- and C-terminal regions, and a variable numbers of near-identical repeats in a central repeat domain, which determines the effector’s specificity (Yang *et al.* 2005; Boch *et al.* 2009; Romer *et al.* 2009). Two hypervariable amino acids in each repeat specify one base pair in the DNA, and thus the array of repeats determines a precise DNA target sequence (Boch *et al.* 2009; Moscou and Bogdanove 2009; Romer *et al.* 2009). Remarkably, this discovery enables the prediction of the exact DNA binding site, and thus the specific genes induced by each TAL effector, with complete accuracy. Moreover, specific recognition sites for each TAL effector can be engineered upstream of suitable resistance genes to engineer protection against *Xanthomonas* infection. Fittingly, plants have already exploited such potential. For example, a binding sequence for the TAL effector AvrBs3 is found in the promoter region of the *Bs3* resistance gene, resulting in conditional expression of *Bs3* in the presence of AvrBs3 (Romer *et al.* 2007). Thus, in contrast to the previous examples, TAL effectors show extreme target specificity and do not seem to act redundantly because several of them are essential for virulence (Gurlebeck *et al.* 2006; Kay and Bonas 2009).

Linking effector activities with effector-triggered immunity

TAL effectors illustrate how bacterial effectors manipulate host gene expression to their advantage, and furthermore how the host can exploit this pathogen-driven recognition event to specify immunity. A similar situation appears to occur for *Pseudomonas* effectors, albeit at the level of protein–protein interactions. As discussed above, *Arabidopsis* RIN4 is targeted by multiple effectors and this has been exploited by the host through protein complexes with immune receptors. RIN4 forms complexes with the NB-LRR proteins RPS2 and RPM1 respectively (Mackey *et al.* 2002, 2003; Axtell and Staskawicz 2003). Degradation of RIN4 by AvrRpt2 activates RPS2, whereas association of AvrB or AvrRPM1 with RIN4 activates RPM1. In another example, PBL kinases such as BIK1 and PBS1 are cleaved by the cysteine protease AvrPphB to inhibit PTI (Zhang *et al.* 2010). However, cleavage of PBS1 is monitored by the NB-LRR protein RPS5, resulting in the activation of ETI (Shao *et al.* 2003). A similarly interesting scenario exists for the effectors AvrPto and AvrPtoB, which target the protein kinase domains of related RKs. In tomato, the NB-LRR protein Prf forms a constitutive complex with Pto kinase (Mucyn *et al.* 2006), which is closely related to the kinase domains of CERK1 and FLS2 (Gohre *et al.* 2008). Thus, AvrPto and AvrPtoB associate with Pto apparently because of their virulence strategy of targeting PRR kinase domains, but in this case they activate the co-evolved Pto/Prf complex. Pto and Prf form a multimeric protein complex that can contain other Pto-like kinases including Fen, Pth2, Pth3 or Pth5 (Gutierrez *et al.* 2010) and recent data suggest that at least one other effector in Pto DC3000 elicits the Prf complex, albeit to a lesser extent than AvrPto and AvrPtoB (Gutierrez *et al.* 2010). It seems highly likely that these examples will be generalised by further discoveries, although it remains to be determined how effector signals, which exert a negative effect on PTI components, can become positive activators of ETI. Interestingly, Ntoukakis and

colleagues recently showed that Pto inactivates AvrPtoB by phosphorylating it within its E3 ligase domain (Ntoukakis et al. 2009). The deactivated AvrPtoB molecule then activates the Prf complex.

New data on cooperation between the *Arabidopsis* TIR-NB-LRR genes *RPS4* and *RRS1* provide an insight into multiple recognition events encoded by a single genetic locus (Birker et al. 2009; Narusaka et al. 2009). These genes are arranged in antiparallel tandem within the major recognition gene complex MRC-J on chromosome 5 (Birker et al. 2009). While it was known previously that *RPS4* confers immunity to *P. syringae* through recognition of the effector AvrRps4 (Gassmann et al. 1999) and *RRS1* recognises the *Ralstonia solanacearum* effector PopP2 (Deslandes et al. 2003), the new data show that these genes are mutually required for both of these resistance specificities, and recognition of the fungus *Colletotrichum higginsianum* as well (Birker et al. 2009; Narusaka et al. 2009). Double mutants in *RPS4* and *RRS1* are not more susceptible than single mutants, suggesting that the encoded proteins act in the same pathway, potentially as members of a protein heterocomplex. Fascinatingly, there are nine other examples of such coordinate NB-LRR gene arrangement in the *Arabidopsis* genome (Narusaka et al. 2009) and numerous other examples of NB-LRR genes working together exist. If the encoded proteins do exist within the same complex, it may be analogous to tomato Prf, which incorporates different Pto-like kinases as part of a multimeric complex to broaden its effector recognition capabilities (Gutierrez et al. 2010). Overall, the data reviewed here reveal some of the micro-evolutionary molecular events that underlie antagonistic co-evolution in the pathogen–plant host relationship.

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