Effector proteins of phytopathogenic bacteria: bifunctional signals in virulence and host recognition

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Phytopathogenic bacteria deliver effectors of disease into plant hosts via a Type III secretion system. These Type III effectors have genetically determined roles in virulence. They also are among the components recognized by the putative receptors of the plant innate immune system. Recent breakthroughs include localization of some of these Type III effectors to specific host cell compartments, and the first dissection of pathogenicity islands that carry them.

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Abbreviations

AD	activating domain
avr	avirulence
HR	hypersensitive response
LRR	leucine-rich repeat
ORF	open reading frame
PAI	pathogenicity island
R	resistance

Introduction

Pathogenic bacteria invading plant tissues must survive a defense system that is very different from the circulating animal immune system. Although mechanisms of bacterial infection of plants and animals were historically thought to be very different, recent advances in the understanding of plant defense mechanisms, bacterial secretion systems, and pathogenicity islands reveals a convergence of infection mechanisms between bacterial pathogens of plants and animals [1,2••,3].

Resistance to bacterial infection by plants is often determined by the presence of a resistance (*R*) gene in plants and an avirulence (*avr*) gene in bacteria [1]. When both of these genetic determinants are present, host defense responses are triggered and pathogen colonization is limited. The absence of either of these genetic components results in disease. Plant defense responses often include cell wall cross-linking, the release of active oxygen species, expression of antimicrobial compounds, and a form of localized cell death, termed the hypersensitive response (HR). Many *avr* genes have been cloned from phytopathogenic bacteria and are diverse in structure [4]. In contrast, the vast majority of *R* genes encode proteins that contain leucine-rich repeat (LRR) domains coupled to a small variety of other putative signaling domains [5••].

The paradoxical presence of bacterial *avr* genes, which by initiating host defense limit pathogen fitness, is resolved by studies demonstrating that various *avr* gene products can function as virulence factors on hosts lacking the corresponding *R* gene [6–8]. Because isolates of phytopathgenic bacteria generally contain only one or a few genes defined by their avirulence function, it can be assumed that virulence is provided by sets of genetically redundant effectors. Either function, initiation of *R*-mediated defenses or enhancement of virulence, requires a bacterially encoded Type III secretion system. [9,10]. Thus, Avr proteins are probable Type III effector proteins. Consistent with this concept, substantial evidence has shown that many Avr proteins can be perceived by *R* genes when expressed in host cells [11–15,16•,17,18•]. Whether Avr and R proteins interact directly to produce *R*-dependent responses is still an open question. We describe here recent studies concerning the distribution of *avr* genes among phytopathogenic bacteria, and how the corresponding Avr proteins are exported from bacteria and perceived by host cells. Previous progress in this topic has also been covered in earlier issues of this journal [3,10,19].

Pathogenicity islands: mechanism for evolution of virulence

Virulence and regulatory genes involved in bacterial pathogenicity are frequently located in blocks on the bacterial chromosome called pathogenicity islands (PAIs). Peculiar to PAIs are DNA sequences indicative of gene mobility such as transposases, flanking direct repeats, or insertion sequence (IS) elements. These regions generally have a different G+C content than their host genome, suggesting acquisition via horizontal gene transfer [20]. PAIs have also been localized to mobilizable plasmids [21] and, interestingly, many *avr* genes are also plasmid localized [22].

A plasmid-encoded PAI was recently isolated from race 7 of *Pseudomonas* pv *phaseolicola* strain 1449B [23••]. Cured of a 154 kb plasmid, this strain is no longer virulent on bean, demonstrating a virulence function for this plasmid. Cosmid clones complementing this loss of virulence were isolated. Sequencing and mutational analysis of these cosmids revealed classic PAI elements: consensus Type III transcriptional regulatory sequences preceding functional virulence genes and avirulence genes, a lower G+C content than the chromosome, insertion sequences homologous to those of *Yersinia pestis* (IS*100*) and a transposase found in *Pseudomonas aeruginosa* (Tn*501*) [23••].

The presence of these mobile elements expanded an earlier computer analysis of database derived *avr* open reading frames (ORFs) This study demonstrated that 14 of 19 published *avr* ORFs are associated with IS elements and transposase terminal repeats [24]. It therefore seems

Table 1

Ectopic expression of *avr* genes in plants lacking corresponding *R* gene.

Avr protein	Delivery system	Recipient plant	Probable subcellular location	Cell phenotype	Pathogen of origin [22]
AvrB	Agrobacterium Stable transformation; inducible promoter	Arabidopsis	Plasma membrane[a]	Chlorosis[a]	*P. syringae* pv. *glycinea*
AvrRpm1	Agrobacterium Stable transformation; inducible promoter	Arabidopsis	Plasma membrane[a]	None	*P. syringae* pv. *maculicola*
AvrE	Agrobacterium	Arabidopsis	ND	Tissue necrosis[b]	*P. syringae* pv. *tomato*
AvrPto	Potato virus X	Tomato, *N. benthamiana*	ND	Necrosis [17]	*P. syringae* pv. *tomato*
AvrPto	Agrobacterium	Arabidopsis	Membrane[c]	No response[b]	*P. syringae* pv. *tomato*
PthA	Agrobacterium and particle bombardment	Citrus	Nuclear [51]	Cell division; canker formation [18•]	*X. citri*
AvrRpt2	Stable transformation; inducible promoter	Arabidopsis	ND	Browning [16•]	*P. syringae* pv. *tomato*
AvrPphE	Agrobacterium	Bean	ND	Weak browning [15]	*P. syringae* pv. *phaseolicola*
AvrPphB	Agrobacterium	Bean	ND	Weak browning [15]	*P. syringae* pv. *phaseolicola*
AvrBs3	Agrobacterium	Pepper	Nuclear [35]	No response [35]	*X. campestris* pv. *glycinea*

[a]Z Nimchuk, S Kjemtrup, E Marois, RT Leister, F Katagiri, JL Dangl, unpublished data. [b]SY He, personal communication. [c]X Tang, personal communication. ND, not determined.

that phytopathogenic bacteria utilize transposon-based mechanism for acquiring or deleting *avr* genes.

Unpublished results from our laboratory suggest that both transposition and plasmid excision can occur in response to selective pressures initiated by host defense responses. *P. syringae*. pv *maculicola* strain M6 (*Psm*M6) contains a chromosomal copy of *avrRpm1* that is recognized by the Arabidopsis *R* gene *RPM1*. Following infection of *RPM1* Arabidopsis (but not *rpm1*-null plants), we observed a novel plasmid containing *avrRpm1* in each colony assayed. Five percent of this bacterial population is also virulent on *RPM1* Arabidopsis, due, in at least one case, to a Tn3 disruption of the *avrRpm1* ORF. This excision event is dependent on *R*-mediated recognition and several additional genetically defined steps in the Arabidopsis disease resistance response pathway (P Marchesini, S Kjemtrup, L Rohmer, J Dangl, unpublished data).

In *P. syringae* pv *pisi*, the *avrRpm1* homologue *avrPpiA1* is either plasmid borne or chromosomally localized in phylogenetically different strains. The *avrPpiA1* chromosomal region from one isolate contains an 8.5kb insert bounded by imperfect direct repeats compared with an *avrPpiA1*-null isolate from the same phylogenic group [25]. Although no known transposase is associated with the characterized repeats, the direct repeat structure suggests that the isolates are related by either a transposon insertion or deletion event.

Delivery of virulence genes: Type III secretion

Type III secretion systems encode host-cell-contact-dependent secretion systems found in many Gram-negative pathogenic bacteria [2••]. This secretion system delivers bacterial virulence effectors to the interior of host cells. Proteins secreted by the Type III system have no apparent common amino acid motif suggestive of a secretion signal. However, amino-terminal frameshift mutated YopN and YopE virulence factors fused to neomycin phosphotransferase II (NPTII) are still secreted by *Yersinia*, indicating that the secretion signal may actually reside in the tertiary 5′ mRNA structure [26]. Interestingly, Type III secretion can be co-translational, as shown for the *Yersinia* virulence factor YopQ. When the Type III secretion pathway is blocked, YopQ is not translated and presynthesized YopQ cannot be exported from the cell [27].

In phytopathogenic bacteria, the genes encoding components of the Type III system were originally designated *hrp* (hypersensitive response and pathogenicity). Those with broad conservation among many Type III systems have been renamed *hrc* (hypersensitive response, pathogenicity and conserved) [28]. The *hrp/hrc* regulon includes regulatory genes, effectors, and structural components of the secretion apparatus [29]. Recent reports show that Avr proteins secreted in a Type III-dependent manner from phytopathogenic bacteria may also require a 5′ mRNA secretion signal. Frameshift mutations in the amino terminus of *avrPto* fused to NPTII still allowed Type III-dependent secretion of the fusion protein into culture medium, suggesting that the secretion signal for AvrPto also resides in the mRNA structure [30•]. Computer modeling suggests that the mRNA 5′ end of Type III secreted proteins can share a common structure composed of the AUG start codon embedded in a stem-loop, although this prediction requires experimental validation.

Type III effectors from phytopathogens can be secreted by heterologous Type III systems indicating the mRNA secretion signal can be recognized by different systems. The *Yersinia* Type III system can secrete the *P. syringae* derived Avr proteins, AvrPto and AvrB, while the *Erwinia chrysanthemi hrp* system recognizes and secretes YopE and YopQ, albeit at lower efficiency than in *Yersinia* [30•]. Confirming these results is a study by Rossier *et al.* [31•] showing that a constitutively active *hrp* system from a *Xanthomonas* strain can recognize and secrete the effector protein PopA from *Ralstonia solanacearum*, the avirulence protein AvrB from *P. syringae*, and indeed, YopE from *Yersinia*. The nonselective nature of these Type III secretion systems points to a common mechanism for injecting effectors of plant and animal pathogens in to host cells.

Translocation of Avr proteins through the *hrp/hrc* system

Culture conditions mimicking the plant apoplast have long been known to induce *hrp*-dependent *avr* expression [32]. Two groups have now described conditions to detect Avr protein secretion. Rossier *et al.* [31•] employed a *Xanthomonas* strain that constitutively expresses *hrp* genes due to a hypermorphic mutation in the regulatory *hrpG* gene. *Hrp*-dependent secretion of Avr proteins could be detected in acidic minimal medium containing bovine serum albumin. Similar conditions for Type III-dependent secretion by *P. syringae* were described by van Dijk *et al.* [33]: an acidic minimal media with a temperature optimum between 18–20°C.

Despite the overwhelming functional data suggesting that the *hrp* system delivers effectors into the plant cell, *hrp*-dependent translocation of Avr proteins into plant cells was difficult to demonstrate. Mudgett and Staskawicz [34••] provide compelling evidence for translocation of *P. syringae* derived AvrRpt2 to the Arabidopsis host cell during infection. Transgenic plants expressing AvrRpt2 proteolytically process the protein to a short form that is not found in bacterial lysates. The same processed form is detected in bacterially infected tissue in a Type III-dependent manner. That the plant protease activity responsible for this cleavage was demonstrated to be intracellular, and not apoplastic, is further confirmation of both AvrRpt2 translocation and an intracellular effector site for its action.

Localization of Avr proteins in host plant cells

What is the fate of *avr*-encoded Type III effector proteins once they are inside a host cell? Indirect evidence suggests that some Avr proteins are directed to specific subcellular locations that in turn may reflect the location of respective host targets. Members of the *Xanthomonas avrBs3* family, including both *avrBs3* and *avrXa10*, contain functional nuclear localization sequences (NLS) required for *R* gene activity, suggesting that recognition occurs in the host nucleus [19,35]. Interestingly, the carboxy-terminal of AvrXa10 contains a transcriptional activating domain (AD) which is active in both yeast and plants [36]. Mutations in this domain that abolish transcriptional activity also abolish avirulence function. This domain can also be replaced by the AD from the VP16 herpes simplex virus protein without abolishing avirulence activity [37••]. These results suggest that AvrXa10-dependent transcriptional activity is necessary for triggering *R* gene function. It is not clear how this transcriptional activity *per se* is required for *R* gene recognition as *Xa10* recognition specificity maps not to the AD but the central region of *avrXa10* [36]. This is consistent with the original demonstration that recognition of the related *avrBs3* gene by *Bs3* also mapped to the central, variable copy number, repeat region [19]. It is possible that the AD may either play a role in Xa10 binding or AvrXa10 stability *in planta*, or recruit AvrXa10 to a nuclear complex containing Xa10.

A class of *P. syringae* Avr proteins may be targeted to the host cell plasma membrane. AvrB, AvrC, AvrRpm1 and AvrPto all contain predicted acylation sites at their amino termini. In eukaryotes, amino-terminal modification such as myristoylation and palmitoylation promote association with membrane compartments [38]. Both AvrB and AvrRpm1 are recognized by RPM1 in Arabidopsis [39]. Unpublished results from our laboratory demonstrate that mutations in the consensus acylation sites of AvrB and AvrRpm1 abolish avirulence functions of both proteins, and the virulence function of AvrRpm1 (Z Nimchuk, E Marois, S Kjemtrup, RT Leister, F Katagiri, J Dangl, unpublished data). In addition, AvrB and AvrRpm1 are myristoylated and plasma membrane localized when expressed *in planta*. Membrane localization correlates with avirulence activity, strongly suggesting that recognition of either AvrB or AvrRpm1 by RPM1 occurs at the plasma membrane. Consistent with this hypothesis, RPM1 is a peripheral plasma membrane protein [40].

Although AvrPto also localizes to a membrane fraction when expressed *in planta* (T Leister and F Katagiri; X Tang, personal communications), a mutant Pto protein that cannot be myristoylated still functions when overexpressed [41]. It is possible that Pto may be recruited to the membrane upon AvrPto translocation or that Pto sequesters incoming AvrPto to the cytoplasm. Alternatively, overexpression of the mutant delivers sufficient quantities of Pto to the membrane, as observed for overexpression of animal G alpha mutants [42].

Interaction with *R* gene products: the receptor complex

The ligand–receptor model predicts that resistant plants recognize incoming Avr proteins via direct interaction with cognate R proteins [43]. In support of this hypothesis, a direct interaction between the tomato Pto kinase and AvrPto was demonstrated in yeast [12,13]. However, the majority of predicted *R* genes are LRR containing products and do not resemble *Pto*. Although the Pto kinase binds AvrPto *in vitro*, *Pto*-mediated resistance genetically requires the LRR *Prf* gene product [44]. *Prf* acts downstream of, or with *Pto* [45]. This raises the possibility that

recognition of AvrPto requires a multi-protein complex. G Martin and A Bogdanove, (personal communication) have identified potential AvrPto/Pto complex participants using a yeast three hybrid system.

Virulence functions of Type III effectors

Although several *avr* genes are now known to contribute to pathogen virulence on susceptible hosts, the mechanism by which they do so and the relevant host targets are unknown. It is interesting that some *avr* genes induce phenotypic effects when expressed in host plants that lack the corresponding *R* allele (see Table 1). In animal–bacterial pathosystems, similar cytotoxic effects on host cells are triggered by Type III effectors, and in many cases these effects identify host targets that are relevant during infection[46–48]. Thus, it is possible that the effects of *avr* gene expression in plant hosts may be indicative of their underlying virulence function. Ectopic expression of *pthA* from *Xanthomonas citri* in susceptible hosts triggers cell enlargement, division and death [18•]. These effects phenocopy disease symptoms attributed to PthA during pathogen infections on these hosts. This finding is supported by the initial observation that AvrBs3, which is related to PthA, also can initiate ectopic cell expansion in susceptible hosts [35]. This is a rich area for further inquiry. For example, will different Type III effectors target a limited set of cellular processes, including inhibition of host defense responses (see below)? Will there be genetically tractable experiments in which to identify host loci controlling the phenotypic effects of these pathogen proteins? Also will the expression of Type III effectors in susceptible hosts result in activation of genes different from those activated during a defense response?

One function of Avr proteins during virulence is likely to be the suppression of inducible host defenses. Curing of the 154 kb plasmid from *P. syringae* pv *phaseolicola* race 7 results in strains that trigger HR-like responses on previously susceptible hosts [23••]. This suggests that one function of the plasmid-born virulence genes is to interfere with host recognition of chromosomal *avr* genes. B Kunkel and co-workers (personal communication) report that *avrRpt2* can increase pathogen aggressiveness on susceptible hosts and that this effect is correlated with a suppression of inducible defense responses. In addition, expression of AvrRpt2 *in planta* is capable of blocking *avrB* or *avrRpm1* activation of *RPM1* [49,50], but does not inhibit other *avr-R* gene combinations in *Arabidopsis* (B Runkel, personal communication). This finding may help elucidate the connections between general inducible defenses and specific *R* gene defense triggering in hosts.

Conclusions

The results and tools described here open avenues to study fundamental questions in plant–pathogen interactions. The existence of PAIs and associated mobile elements is evidence that host range of phytopathogens is molded by horizontal gene transfer. Characterization of Type III regulated ORFs on PAIs should provide insights into virulence mechanisms. *In vitro* secretion assays for Type III systems will undoubtedly help elucidate regulatory mechanisms and identify novel effector proteins. Expression of Type III effector proteins in host plant cells will define subcellular sites of action, identify targets in susceptible hosts and help understand *R*-mediated recognition of Avr proteins. These studies will reveal both the similarities and differences between bacterial pathogenesis of animals and plants. They will also provide novel protein probes of normal host cellular functions.

Note added in proof

An important recent demonstration of direct transfer of chromosomal copper resistance and *hrp* genes from a donor *X. axonopodis* pv. *vesicatoria* to a recipient strain of the same species provides compelling evidence for horizontal transmission. Interestingly, the frequency of gene transfer was greater from *in planta* matings than from plate matings, implicating a plant factor involvement with chromosomal transfer [52••].

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