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Getting across—bacterial type III effector proteins on their way to the plant cell

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Pathogenicity of most Gram-negative bacterial plant pathogens depends on *hrp* (hypersensitive response and pathogenicity) genes, which control the ability to cause disease and to elicit specific defense responses in resistant plants. *hrp* genes encode a specialized type III secretion (TTS) system that mediates the vectorial delivery of bacterial effector proteins across both bacterial membranes as well as across the eukaryotic plasma membrane into the host cell cytosol. One well-studied effector protein is AvrBs3 from *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of bacterial spot in pepper and tomato. AvrBs3 induces hypertrophy symptoms in susceptible plants and triggers a resistance gene-specific cell death reaction in resistant plants. Intriguingly, AvrBs3 has characteristic features of eukaryotic transcription factors, suggesting that it modulates the host's transcriptome. Here, we discuss the TTS system of *X.campestris* pv. *vesicatoria* in the light of current knowledge on type III-dependent protein secretion in plant pathogenic bacteria.

Keywords: AvrBs3/*hrp* genes/pathogenicity island/PIP box/secretion

Introduction

Plants provide an attractive nutrient reservoir and ecological niche for bacterial pathogens. In most higher plants, bacterial colonization leads to a variety of severe diseases. However, disease is the exception rather than the rule since most plants possess a battery of defense mechanisms that repel invading microbes. Therefore, Gram-negative plant pathogenic bacteria have evolved sophisticated strategies to colonize their host plants. They enter the plant through natural openings such as stomata, or wounds, and multiply in the intercellular spaces of the tissue at the expense of the host.

Over the past two decades, genetic and molecular studies unraveled important mechanisms underlying bacterial pathogenicity. Essential for the molecular cross-talk between pathogens and their host plants is a specialized protein delivery system, the type III secretion (TTS) system. TTS systems are conserved in plant and animal pathogenic bacteria and mediate the vectorial delivery of bacterial effector proteins into the host cell (Hueck, 1998; Cornelis and Van Gijsegem, 2000). In plant pathogens, TTS systems are encoded by *hrp* (hypersensitive response and pathogenicity) genes, essential determinants of bacterial pathogenicity that control the ability to multiply in susceptible hosts and to cause disease (Alfano and Collmer, 1997). In addition, *hrp* genes are required to induce the hypersensitive response (HR), a rapid localized programmed death of plant cells at the infection site, in resistant host and in non-host plants (Klement, 1982). The HR is part of the plant's innate immune response that halts bacterial ingress. Induction of the HR is due to the specific recognition of a bacterial effector protein [designated avirulence (Avr) protein] by a corresponding plant resistance (R) protein (Flor, 1971; Table I).

Among the model organisms for the molecular and genetic characterization of host–plant interactions and the functional analysis of TTS systems are *Erwinia amylovora*, *Ralstonia solanacearum*, pathovars (pv.) of *Pseudomonas syringae* and species (spp.) of *Xanthomonas*, all infecting important crop plants. The pathovar designation refers to differences in the host range of the bacteria. For some of these bacteria, the genome sequence has become available recently, initiating a new era in molecular plant pathology (Da Silva *et al*., 2002; Salanoubat *et al*., 2002; www.tigr.org). Our laboratory studies *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of bacterial spot in pepper and tomato plants, which is the focus of this review.

The *hrp* pathogenicity island—genetic requisite for effector protein traffic

Gram-negative bacteria utilize different protein secretion systems to transport proteins across the inner and outer membrane. Among the six main groups of secretion systems, TTS systems exhibit the most complex architecture (Thanassi and Hultgren, 2000). Around 20 proteins are involved in the formation of a membrane-spanning secretion apparatus, which is associated with an extracellular filamentous structure (Hueck, 1998; see below).

Type III-mediated protein secretion into the extracellular medium was discovered initially in the animal pathogen *Yersinia enterocolitica* (Heesemann *et al*., 1984). However, the first genes encoding components of the TTS system were identified by the analysis of non-pathogenic mutants of the plant pathogens *P.syringae* pv. *syringae* and *P.syringae* pv. *phaseolicola* (Niepold *et al*., 1985; Lindgren *et al*., 1986). Except for *Agrobacterium* spp., *hrp* genes are present in all Gram-negative biotrophic plant pathogens and are generally organized in large clusters comprising >20 genes (Boucher *et al*., 1987; Steinberger and Beer, 1988; Barny *et al*., 1990; Arlat *et al*., 1991; Bonas *et al*., 1991). Based on similarities in *hrp* gene organization and regulation, plant pathogenic bacteria have been classified into two groups, group I (*E.amylovora* and *P.syringae*) and group II (*R.solanacearum* and species

of *Xanthomonas*) (Alfano and Collmer, 1996). At least nine *hrp* genes (termed *hrc* for *hrp* conserved) are conserved in both groups and encode components of the TTS system, which are also present in animal pathogenic bacteria (Bogdanove *et al*., 1996; He, 1998; Hueck, 1998). Hrc proteins presumably constitute the core components of the secretion apparatus in the inner and outer membrane. With the exception of HrcC—the best studied Hrc protein, which belongs to the secretin family of outer membrane proteins—Hrc proteins share sequence similarities with flagellar assembly components. The flagellar assembly apparatus serves as a protein export system and probably represents an evolutionary ancestor of the TTS system (Hueck, 1998; Macnab, 1999; Aizawa, 2001; Young and Young, 2002).

In contrast to conserved *hrp* genes, the precise role of non-conserved *hrp* genes remains to be investigated. Genetic studies of *X.campestris* pv. *vesicatoria* revealed that type III secretion requires at least six non-conserved *hrp* genes, some of which encode type III-secreted proteins, e.g. HrpB2 (Rossier *et al*., 2000; Table II). The *hrp* region also contains so-called *hrp*-associated (*hpa*) genes (Figure 1), which are not essential for bacterial pathogenicity but contribute to the interaction with the host plant (Huguet *et al*., 1998; Noël *et al*., 2002; O.Rossier, D.Büttner and U.Bonas, unpublished data).

How did *hrp* gene clusters evolve? Genes involved in bacterial virulence often are located in regions that show characteristics of pathogenicity islands. These DNA regions usually are flanked by direct repeats, insertion sequence (IS) elements, tRNA genes and/or genes for integrases and transposases. Pathogenicity islands often differ in G + C content from the genomic DNA, indicating horizontal gene transfer (Hacker and Kaper, 2000). In *X.campestris* pv. *vesicatoria*, mobility of the *hrp* region has indeed been observed (Basim *et al*., 1999). Furthermore, sequence analyses of DNA regions flanking the *hrp* gene cluster revealed the presence of an IS-like element and putative effector genes with lower G + C content than the genomic DNA (Noël *et al*., 2002; Figure 1).

Typical features of pathogenicity islands are also present in DNA sequences flanking the *hrp* gene cluster of *P.syringae*. Here, the region adjacent to *hrpK* has a lower G + C content and contains sequences homologous to IS elements, transposases and tRNA genes. Interestingly, the genes located in this region, termed exchangeable effector locus (EEL), vary in pathovars of *P.syringae* (Alfano *et al*., 2000; one example is given in Figure 1).

Table I. *R* gene-specified pathogen recognition according to gene-for-gene interactions[a]

Pathogen genotype	Plant reaction	
	Host plant genotype[b] *R1/R1* or *R1/r1*	*r1/r1*
avr1	HR[d]	Disease
–[c]	Disease	Disease

[a]Gene-for-gene hypothesis (Flor, 1971).
[b]*R1*, resistance locus allowing recognition of a corresponding avirulence (*avr*) gene (designated *avr1*). Most resistance (*R*) genes are single dominant genes. *r1* refers to the absence of a functional *R1* allele.
[c]The avirulence gene is absent or mutated, resulting in loss of recognition by plants carrying the corresponding *R* gene.
[d]HR, hypersensitive reaction.

Entering the plant—green light for *hrp* gene expression

Type III secretion is a regulated process. Genes encoding components of the secretion apparatus are not constitutively expressed but activated *in planta* and in minimal media mimicking the environmental conditions present in the plant apoplast (Lindgren, 1997). Proteins involved in

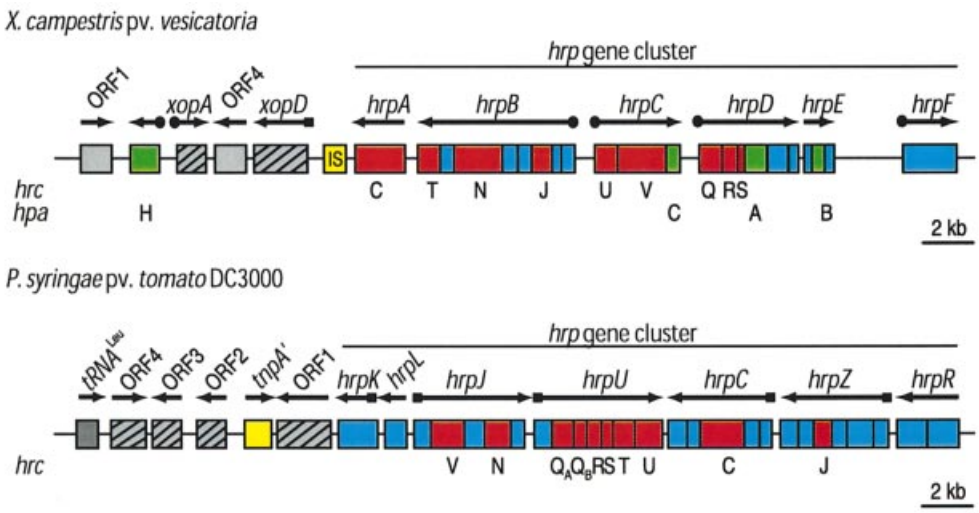

Fig. 1. Schematic overview of the *hrp* gene clusters and the left flanking regions from *X.campestris* pv. *vesicatoria* (group II) and *P.syringae* pv. *tomato* DC3000 (group I). The regions contain *hrp*, *hrc* and *hpa* genes (represented in blue, red and green, respectively). Arrows indicate the direction of transcription. Black dots and squares refer to the presence of PIP and *hrp* boxes, respectively. Hatched regions correspond to sequences with low G + C content; yellow regions refer to mobile genetic elements.

Table II. *Xanthomonas campestris* pv. *vesicatoria* type III-secreted proteins

Protein[a]	Characteristics/homology[b]	Expression[c]	References
Components of the TTS apparatus			
HrpB2‡	Extracellular component of the TTS system	Induced; PIP box	Wengelnik and Bonas (1996); Rossier *et al.* (2000)
HrpE1‡	Major Hrp pilus subunit	Basal expression, induction	Wengelnik and Bonas (1996); Rossier (1999); T.Ojanen-Reuhs and U.Bonas (unpublished data)
HrpF	Translocon protein	Induced; PIP box	Wengelnik and Bonas (1996); Rossier *et al.* (2000); Büttner *et al.* (2002)
Xops[d]			
√XopA	Hpa1 (*X.oryzae* pv. *oryzae*)	Induced; PIP box	Noël *et al.* (2002)
XopB	AvrPphD (*P.syringae* pv. *phaseolicola*)	Induced	Noël (2001); Noël *et al.* (2001)
XopC		Induced	Noël (2001); Noël *et al.* (2001)
XopD	PsvA (*P.syringae* pv. *eriobotryae*)	Induced; *hrp* box	Noël *et al.* (2002)
XopJ	AvrRxv/YopJ family; putative cysteine protease	Induced	Noël (2001); Noël *et al.* (2001)
√HpaA	NLS	Induced; PIP box	Wengelnik and Bonas (1996); Huguet *et al.* (1998)
AvrBs1*	AvrA (*P.syringae* pv. *glycinea*)	Constitutive	Ronald and Staskawicz, (1988); Escolar *et al.* (2001)
√AvrBs2*	Agrocinopine synthase (*A.tumefaciens*); phosphodiesterase (*E.coli*)	ND	Kearney and Staskawicz (1990); Swords *et al.* (1996); Mudgett *et al.* (2000)
√AvrBs3*	NLS; AAD; AvrBs3 family	Constitutive[e]	Van den Ackerveken *et al.* (1996); Rossier *et al.* (1999); Szurek *et al.* (2001); Marois *et al.* (2002)
AvrBs4*	NLS; AAD; AvrBs3 family	Constitutive[e]	Bonas *et al.* (1993); Ballvora *et al.* (2001)
AvrBsT*	AvrRxv/YopJ family; putative cysteine protease	Constitutive	Escolar *et al.* (2001)
AvrRxv	AvrRxv/YopJ family; putative cysteine protease	Constitutive; PIP box	Ciesiolka *et al.* (1999); Rossier *et al.* (1999)
AvrXv3*	AAD	Induced; PIP box	Astua-Monge *et al.* (2000a)
AvrXv4	AvrRxv/YopJ family; putative cysteine protease	ND; PIP box	Astua-Monge *et al.* (2000b)

[a]‡, essential for type III secretion *in vitro*; √, virulence activity demonstrated; *, indicates ability of Avr proteins to induce the HR upon transient expression in resistant plants.
[b]AAD, acidic activation domain; NLS, nuclear localization signal; Yop, *Yersinia* outer protein.
[c]Expression *in planta* or under *hrp* gene-inducing conditions. ND, not determined; PIP, plant-inducible promoter.
[d]Xops, *Xanthomonas* outer proteins, include type III-secreted proteins with unknown destination as well as avirulence (Avr) proteins; Hpa, *hrp* associated.
[e]Recent *in vitro* expression experiments indicate that *hrpG** leads to a 2- to 3-fold increase in expression (U.Bonas *et al*., unpublished data).
√, virulence activity demonstrated.
*, indicates ability of Avr proteins to induce the HR upon transient expression in resistant plants.

hrp gene regulation vary in the different groups of plant pathogens. In *X.campestris* pv. *vesicatoria*, *hrp* gene expression is controlled by HrpX, an AraC-type transcriptional activator (Wengelnik and Bonas, 1996). In minimal medium or *in planta*, the expression of *hrpX* is activated by HrpG, a transcriptional activator of the OmpR family of two-component regulators (Wengelnik *et al*., 1996; Figure 2A). Recent transcriptome analysis revealed that HrpG, in most cases via HrpX, controls a genome-wide regulon including *hrp* genes and genes encoding *Xanthomonas* outer proteins (Xops; Wengelnik and Bonas, 1996; Astua-Monge *et al*., 2000a; Noël *et al*., 2001, 2002).

Interestingly, one of the *xop* genes, *xopD*, contains an *hrp* box-like motif in the promoter region (Figure 1; Table II). The *hrp* box is a conserved consensus sequence which was identified in promoters of *hrp* and effector genes in *P.syringae*. It presumably provides the binding site for HrpL, a member of the extracytoplasmic function family of sigma factors (Innes *et al*., 1993; Xiao and Hutcheson, 1994; Xiao *et al*., 1994; Fouts *et al*., 2002). In *X.campestris* pv. *vesicatoria*, however, expression of *xopD* is controlled by HrpG and HrpX (Noël *et al*., 2002). *xopD* encodes a putative type III effector protein with homology to the virulence factor PsvA from *P.syringae* pv. *eriobotryae* (Noël *et al*., 2002; Table II). The presence of an *hrp* box in the *xopD* promoter and the low G + C content of *xopD* support the hypothesis that genes involved in bacterial virulence might have been acquired during evolution by horizontal gene transfer.

Many *hrpX*-regulated genes of *X.campestris* pv. *vesicatoria* contain a PIP (plant-inducible promoter, consensus TTCGC-N_{15}-TTCGC) box in their promoter regions. This sequence motif might be involved in the HrpX-mediated gene regulation (Fenselau and Bonas, 1995; Wengelnik and Bonas, 1996; Noël *et al*., 2002). However, there are also *hrpX*-independent promoters that contain a PIP box, e.g. *avrRxv* (Table II), indicating that the PIP box is not sufficient to confer inducibility by HrpX. In addition, the promoters of several *xop* genes that are controlled by HrpG and HrpX do not contain PIP boxes (Table II). Thus, it remains speculative whether the PIP box serves as a control element. So far, direct binding of HrpX to PIP box-containing promoter sequences could not be demonstrated (L.Escolar and U.Bonas, unpublished data).

PIP box-like motifs have also been identified in *Xanthomonas axonopodis* pv. *citri* and *X.campestris* pv. *campestris* in the promoters of *hrp* genes as well as genes encoding putative proteins with type II signal peptides and sequence homologies to cell wall-degrading enzymes, proteases and an iron receptor (Da Silva *et al*., 2002). Furthermore, PIP box-like promoter sequences have been

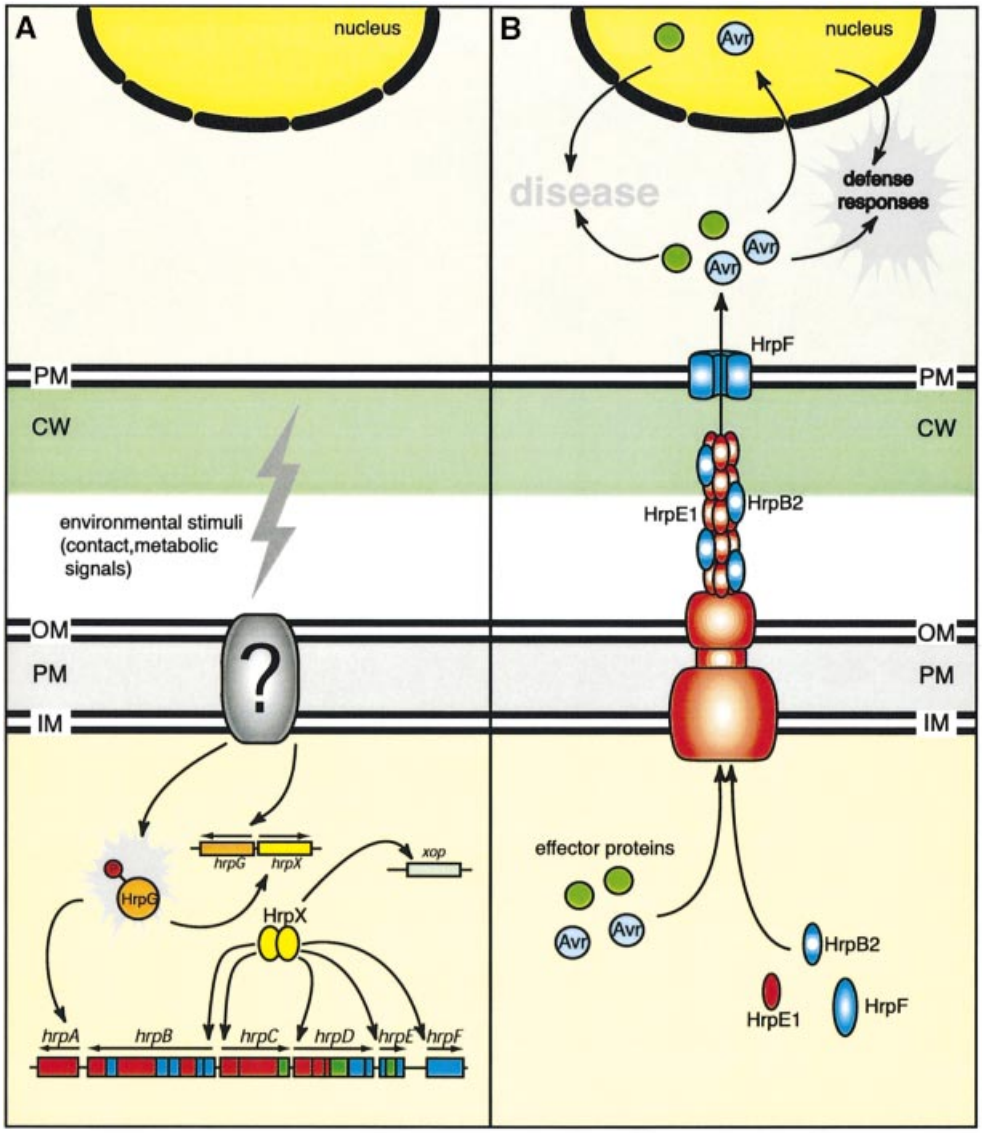

Fig. 2. Model for *hrp* gene regulation and type III secretion in *X.campestris* pv. *vesicatoria*. (**A**) A so far uncharacterized signal transduction system in the bacterial envelope (indicated by a question mark) senses environmental stimuli and transduces the signal to HrpG. HrpG activates the expression of *hrpA* and, via HrpX, the expression of *hrpB–hrpF* as well as of a number of *xop* genes. (**B**) Expression of *hrp* genes is essential for the formation of the TTS apparatus, which spans both bacterial membranes and mediates secretion of Hrp and effector proteins. The TTS apparatus is associated with the Hrp pilus, which presumably spans the cell wall (200 nm thick; not drawn to scale). The major subunit of the Hrp pilus is HrpE1. Translocation of effector proteins across the plant plasma membrane requires HrpF, the putative pore-forming component of the type III translocon. Effector proteins are targeted to different locations in the plant cell and presumably modulate cellular processes leading to disease symptom formation in susceptible plants. In resistant plants, effector proteins (designated Avr proteins) can be recognized and trigger the activation of specific defense responses. CW, cell wall; IM, inner membrane; OM, outer membrane; PM, plasma membrane.

identified in *R.solanacearum*, upstream of *hrp* transcription units, the *popA* gene and several *avr* gene homologs (Fenselau and Bonas, 1995; Wengelnik and Bonas, 1996; Salanoubat *et al*., 2002). In *R.solanacearum*, *hrp* genes are controlled by HrpG and HrpB, which are homologous to HrpG and HrpX, respectively, from *X.campestris* pv. *vesicatoria* (Genin *et al*., 1992; Brito *et al*., 1999). In *R.solanacearum*, the outer membrane protein PrhA (plant regulator of *hrp* genes) presumably is on top of the regulatory cascade leading to *hrp* gene expression. PrhA is homologous to TonB-dependent siderophore receptors and acts as a sensor for a non-diffusible molecule present in the plant cell wall (Marenda *et al*., 1998; Brito *et al*., 1999, 2002; Aldon *et al*., 2000). In contrast to *R.solanacearum*, the receptor(s) in *X.campestris* pv. *vesicatoria* that transmits external stimuli into the bacterial cell is still unknown (Figure 2).

Hrp pilus—tunnel to the host cell

TTS systems have been visualized in the animal pathogens *Salmonella typhimurium*, *Shigella flexneri* and *Escherichia coli*, and show striking morphological similarities to flagellar basal bodies: a membrane-embedded complex is associated with an extracellular hollow struc-

ture, the needle (Kubori *et al*., 1998; Tamano *et al*., 2000; Blocker *et al*., 2001; Sekiya *et al*., 2001).

Type III-dependent surface appendages have also been identified in plant pathogenic bacteria, i.e. *P.syringae* pv. *tomato*, *E.amylovora*, *R.solanacearum* and *X.campestris* pv. *vesicatoria*. These so-called Hrp pili have a similar diameter (6–8 nm), but are considerably longer than the needles of animal pathogens (Roine *et al*., 1997; Van Gijsegem *et al*., 2000; Hu *et al*., 2001; Jin *et al*., 2001; T.Ojanen-Reuhs and U.Bonas, unpublished data). Since Hrp pili can extend to a length of several micrometers, they have been proposed to cross the plant cell wall (Romantschuk *et al*., 2001; Figure 2). In *R.solanacearum* and *P.syringae* pv. *tomato*, the pilin, which is the major subunit of the Hrp pilus, is required for type III secretion *in vitro* (Van Gijsegem *et al*., 2000; Wei *et al*., 2000). Recent immunocytochemical analyses in *E.amylovora* and *P.syringae* pv. *tomato* elegantly demonstrated that Hrp pili serve as conduits for secreted proteins (Brown *et al*., 2001; Jin and He, 2001; Jin *et al*., 2001; Li *et al*., 2002). So far, there are no indications that Hrp pili also mediate bacterial contact with the host cell. In *R.solanacearum*, mutation of *hrpY*, the gene encoding the major pilus subunit, does not affect attachment of the bacteria to cultured plant cells (Van Gijsegem *et al*., 2000).

Getting in touch—the type III translocon

Translocation across the eukaryotic plasma membrane probably requires the presence of type III-secreted bacterial proteins that form the type III translocon, a channel-like complex in the host plasma membrane (Büttner and Bonas, 2002). Putative components of the translocon have been described mainly in animal pathogens whereas they have not been identified so far in most plant pathogenic bacteria. To our knowledge, HrpF from *X.campestris* pv. *vesicatoria* is the first known candidate for a type III translocon protein in bacterial plant pathogens. Mutant studies revealed that HrpF, which is secreted by the TTS system, is dispensable for type III secretion *in vitro* but essential for the interaction with the plant (Rossier *et al*., 2000; Büttner *et al*., 2002). *hrpF* mutants are not able to grow and cause disease in susceptible plants and to induce the HR in resistant plants. When tested in artificial lipid bilayer systems, HrpF induced pore formation, suggesting that it might be the channel-forming core component of the type III translocon (Büttner *et al*., 2002; Figure 2). Pore-forming activity has been demonstrated for the putative type III translocon proteins LcrV and PcrV from *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*, respectively, which do not show any sequence similarity to HrpF (Holmström *et al*., 2001).

In animal pathogenic bacteria, observations of protein–protein interactions between putative translocon proteins suggest that the type III translocon is a heterogeneous protein complex. For instance, LcrV presumably interacts with YopB and YopD to build a functional translocon (Sarker *et al*., 1998). In *X.campestris* pv. *vesicatoria*, it remains to be investigated whether additional proteins besides HrpF are involved in the formation of the type III translocon. So far, studies to identify HrpF interaction partners failed since HrpF is a 'sticky' protein, making it difficult to show interaction specificity (D.Büttner and U.Bonas, unpublished data).

Carte d'accès—recognition by the TTS system

The mechanisms that control type III secretion *in planta* are still unknown. In *Erwinia* spp., *P.syringae* and *R.solanacearum*, several type III-secreted proteins could be detected in the culture supernatant after incubation of the bacteria in *hrp* gene-inducing medium (e.g. Gaudriault *et al*., 1997; Mudgett and Staskawicz, 1999; Van Gijsegem *et al*., 2000).

In *X.campestris* pv. *vesicatoria*, the isolation of a point mutation in *hrpG* (E44K, designated *hrpG**), which leads to constitutive expression of *hrp* genes, was key for the establishment of an *in vitro* secretion assay (Rossier *et al*., 1999; Wengelnik *et al*., 1999). However, expression of the *hrp* genes is not sufficient to trigger type III secretion. The identification of secreted proteins requires the incubation of *hrpG** bacteria in acidic minimal medium, which probably mimicks the plant's apoplast. Interestingly, the *X.campestris* pv. *vesicatoria* TTS system also secretes heterologous proteins such as PopA from *R.solanacearum*, AvrB from *P.syringae* and YopE from *Y.pseudotuberculosis*, indicating that the secretion signal is conserved among plant and animal pathogenic bacteria (Rossier *et al*., 1999).

What is the nature of the secretion signal in proteins traveling the TTS systems? It has been proposed that the signal resides in the N terminus of the secreted proteins. In *Yersinia* spp., the first 11–17 amino acids of *Yersinia* outer proteins (Yops) are sufficient to drive the type III-dependent secretion of a reporter protein (Sory *et al*., 1995; Schesser *et al*., 1996; Lloyd *et al*., 2001b). Similarly, in *X.campestris* pv. *vesicatoria*, the first 28 amino acids of AvrBs2 contain a functional secretion signal (Mudgett *et al*., 2000). Type III-secreted proteins in both plant and animal pathogens do not share any sequence conservations in their N termini. However, comparative sequence analyses of multiple type III-secreted proteins of *P.syringae* pathovars revealed similarities in their N-terminal amino acid composition, including a high content of serine residues (on average 16–18%; Guttman *et al*., 2002; Petnicki-Ocwieja *et al*., 2002). In *X.campestris* pv. *vesicatoria*, the serine content within the first 25 amino acids of known TTS substrates varies between 8% (HrpB2) and 32% (HrpF). This is significantly higher than the serine content in the N termini of non-secreted components of the TTS system (betweeen 0%, as in HrcN, and 12%, as in HrcT).

Since frameshift mutations in the N-terminal coding sequence did not abolish type III secretion of a reporter protein in *Y.enterocolitica*, the secretion signal was also predicted to reside in the 5′ region of the mRNA (Anderson and Schneewind, 1997). This hypothesis, which assumes a co-translational secretion, is, however, discussed controversially in the field. For instance, the *Yersinia* YopE and YopH proteins are expressed even in the absence of a functional TTS system. In addition, mutations in YopE resulting in an altered mRNA structure did not abolish its type III secretion (Lloyd *et al*., 2001b).

The situation is complicated further by the finding that several effector proteins from animal pathogens require specific chaperones for type III secretion and translocation (Bennett and Hughes, 2000; Lloyd *et al*., 2001a). Recently, TTS chaperones have also been identified in plant pathogenic bacteria. DspB from *E.amylovora* and ShcA from *P.syringae* are essential for the stability and/or secretion of the pathogenicity factor DspA and the effector protein HopPsyA, respectively (Gaudriault *et al*., 2002; van Dijk *et al*., 2002).

Quo vadis—type III-secreted proteins

Harpins

The first proteins known to be secreted by the TTS system of bacterial plant pathogens were the harpins; HrpZ from *P.syringae* and PopA from *R.solanacearum* (He *et al*., 1993; Arlat *et al*., 1994). Harpins are small, heat-stable, glycine-rich proteins that lack cysteines and elicit a necrosis-like reaction when infiltrated into non-host plants (Wei *et al*., 1992; He *et al*., 1993; Arlat *et al*., 1994; Alfano *et al*., 1996; Gaudriault *et al*., 1998). Interestingly, HrpZ from *P.syringae* was found to bind to the plant plasma membrane and to form ion-conducting pores in artificial lipid bilayers (Lee *et al*., 2001a,b). However, the role of harpins is not well understood. In most cases, a contribution to bacterial virulence could not be demonstrated. Only in *E.amylovora*, a mutation of the harpin gene *hrpN* results in the formation of reduced disease symptoms in susceptible plants (Wei *et al*., 1992; Barny, 1995).

Effector proteins

The best studied effector proteins are the products of *avr* genes, which were first identified genetically without knowing that they encode TTS substrates. Since the isolation of the first *avr* gene, *avrA* from *P.syringae* pv. *glycinea* (Staskawicz *et al*., 1984), >40 bacterial *avr* genes have been identified, mainly in species of *Pseudomonas* and *Xanthomonas* (Vivian and Arnold, 2000). As mentioned above, *avr* genes trigger an *R* gene-specific plant defense reaction which often culminates in the HR. The HR phenotype is easy to follow and has been instrumental in the dissection of both bacterial pathogenicity and specific defense reactions in the plant. In the absence of the corresponding *R* gene, no recognition occurs and the infection leads to disease. There is accumulating evidence that Avr proteins probably act as virulence factors, manipulating host cellular processes for the pathogen's benefit and thus contributing to bacterial fitness and/or symptom formation in susceptible plants (White *et al*., 2000). However, it should be emphasized that mutations in putative effector genes often do not affect bacterial virulence under laboratory conditions, indicating that they play a minor role or have redundant functions.

Until recently, type III-dependent delivery of bacterial effector proteins into the host cell has not been proven. Strong indirect evidence for translocation was provided by the fact that *avr* genes induced an *R* gene-specific HR when expressed inside the plant cell (Bonas and Van den Ackerveken, 1997; Cornelis and Van Gijsegem, 2000). Furthermore, several type III-secreted proteins from plant pathogens contain typical eukaryotic features, indicating an activity inside the host cell (White *et al*., 2000). For instance, the putative myristoylation motifs of several Avr proteins in pathovars of *P.syringae* suggest a localization to the plant plasma membrane, which has indeed been shown for AvrB and AvrRpm1 (Nimchuk *et al*., 2000). In these proteins, the myristoylation motifs are crucial for the avirulence function. Further support for the hypothesis of type III-dependent delivery of bacterial effector proteins into the plant cell was provided by the analysis of the effector protein AvrBs2 from *X.campestris* pv. *vesicatoria*, which was fused translationally to an adenylate cyclase reporter from *Bordetella pertussis* (Casper-Lindley *et al*., 2002). Recently, the direct detection of a bacterial effector protein in the plant cell has been reported: AvrBs3 from *X.campestris* pv. *vesicatoria* could be visualized in nuclei of infected plant cells, using an AvrBs3-specific antibody (Szurek *et al*., 2002; see below).

Arrival—AvrBs3 localizes to the plant cell nucleus

Characteristic eukaryotic protein motifs are also present in members of the AvrBs3 protein family in species of *Xanthomonas* (Gabriel, 1999; Lahaye and Bonas, 2001). AvrBs3-like proteins are highly homologous (90–97% amino acid sequence identity) and all contain C-terminal nuclear localization signals and an acidic activation domain, which are features of eukaryotic transcription factors (Yang and Gabriel, 1995; Van den Ackerveken *et al*., 1996; Zhu *et al*., 1998, 1999; Yang *et al*., 2000; Ballvora *et al*., 2001; Szurek *et al*., 2001). Differences between the family members are restricted mainly to the central protein region, which consists of 13.5–25.5 nearly perfect 34-amino-acid repeats (Lahaye and Bonas, 2001).

The AvrBs3 protein family is named after the first isolated member, AvrBs3 from *X.campestris* pv. *vesicatoria* (Bonas *et al*., 1989). AvrBs3 is one of the few Avr proteins for which a role in symptom formation could be demonstrated. In susceptible host plants, AvrBs3 induces hypertrophy, an enlargement of mesophyll cells (Marois *et al*., 2002). Since the induction of hypertrophy symptoms depends on functional nuclear localization signals and the acidic activation domain, we speculate that AvrBs3 acts as a transcription factor in the host cell nucleus. The nuclear localization signals probably provide the admission ticket for AvrBs3 to use the host's protein traffic road into the nucleus. Indeed, yeast two-hybrid studies and *in vitro* pull-down assays revealed that AvrBs3 interacts with pepper importin α which, together with importin β, mediates nuclear protein import (Görlich *et al*., 1995; Szurek *et al*., 2001; Figure 3). Immunocytological analyses demonstrated that the nuclear localization signals are essential for the targeting of AvrBs3 to nuclei of infected plant cells (Szurek *et al*., 2002).

The hypothesis that AvrBs3 acts as a transcription factor is supported by transcriptome analyses of infected susceptible pepper plants. cDNA-AFLP (cDNA-amplified fragment length polymorphism) studies unraveled AvrBs3-induced genes, designated *upa* (up-regulated by AvrBs3; Marois *et al*., 2002). Sequence analyses revealed that several *upa* genes show homologies to auxin-induced

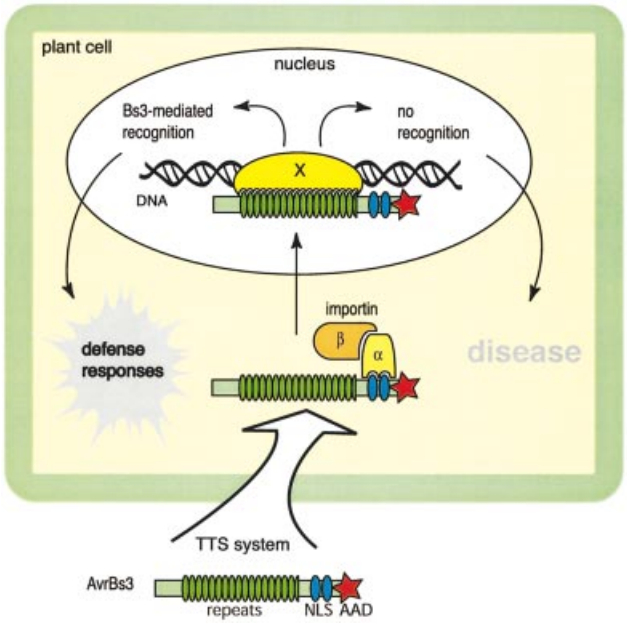

Fig. 3. Proposed model for the molecular mechanisms underlying virulence and avirulence activity of AvrBs3 from *X.campestris* pv. *vesicatoria*. Characteristic features of AvrBs3 are the central 17.5 nearly identical 34 amino acid repeats, two functional C-terminal nuclear localization signals (NLSs) and an acidic activation domain (AAD). Delivery of AvrBs3 into the host cell is mediated by the TTS system. In the plant cell, the NLSs bind to importin α, which together with importin β targets AvrBs3 to the plant cell nucleus. Direct or indirect (via a target protein X) interaction of AvrBs3 with the plant DNA leads to the modulation of the host's transcriptome and presumably results in hypertrophy, a disease symptom in susceptible plants. In resistant plants, specific plant defense responses are induced upon recognition of AvrBs3 by the R protein Bs3 (Bs, bacterial spot).

and expansin-like genes that usually play a role in cell enlargement.

Whether AvrBs3 induces gene expression with the aid of plant transcription factors or directly targets plant promoter sequences is not known (Figure 3). Support for a direct interaction of AvrBs3-like proteins with the host DNA comes from recent studies on AvrXa7, an AvrBs3 homolog from the rice pathogen *Xanthomonas oryzae* pv. *oryzae*, which directly binds to AT-rich DNA sequences (Yang *et al*., 2000).

Perspectives

In the past decade, tremendous progress has been made in dissecting the plethora of type III-secreted proteins in plant pathogenic bacteria. Genetic and biochemical studies have led to the identification of a variety of effector proteins that travel the TTS system, the bacterial main road into the host cell. The next major challenge is the functional analysis of effector proteins: what are their targets in the plant and how do they interfere with host cellular processes? Expression of individual effector proteins in plant cells followed by transcriptome analysis and biochemical approaches will advance our understanding of the molecular processes in infected plant cells. Interdisciplinary approaches and comparative analyses of different pathogen–host systems should not only provide a better understanding of the molecular basis of bacterial pathogenicity but also give us some clues about plant defense and last, but not least, solutions for disease management.

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