

REVIEW ARTICLE

Targeting injectisomes of virulence: Benefits of thirty years of research on bacterial secretion systems, to crop protection

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Summary The discovery in the mid-80s of contiguous gene clusters that were later shown to code for a novel protein secretion system has been a milestone in plant pathology and the study of plant-bacterial interactions. This system, named type III secretion system (T3SS), is dedicated to the translocation of virulence-related proteins, called effectors, from the pathogen to the host and is common among phytopathogenic, zoopathogenic and symbiotic bacteria. Bacterial pathogens overcome multiple layers of plant defense, both preformed barriers and inducible mechanisms. To accomplish this they deploy sophisticated molecular devices to secrete selected sets of proteins either to the intercellular spaces or directly inside the plant cell. Such proteins enable pathogens to avoid recognition, block induction of immune responses and/or interfere with the defense signaling network. Recent developments in molecular biology facilitated research on these interactions and rendered the interkingdom trafficking of proteins a key element of pathogenicity, virulence and host specificity of Gram-negative bacterial pathogens. While basic research on phytobacterial pathogens has progressed, little has changed on the crop protection schemes against bacterial infections. In this review, we summarize the latest findings on the different levels at which contact-dependent protein secretion from Gram-negative bacteria subverts and/or manipulates plant immunity. Additionally, we focus on the biotechnological applications which have emerged from basic research on phytobacterial T3SS and its cognate effectors, ranging from bacterial diagnostics, plant resistance and anti-virulence chemicals to toolkits for gene targeting in plants.

Additional keywords: Diagnostics, effector protein, *hrp*, resistance, type III secretion

Introduction

Bacteria have evolved different strategies to adapt and colonize specific niches, including the plant phyllosphere and rhizosphere. Dependence on the host for survival has lead bacterial species to adapt to a symbiotic or a pathogenic lifestyle, the latter including strategies to proliferate, disperse and overcome plant defenses in order to be suc-

cessful pathogens. For this purpose, pathogens deploy an arsenal of molecular weapons, of which toxins, phytohormones, cell wall degrading enzymes, lipo- and exopolysaccharides were identified and characterized during the 60s and 70s. In the same period, scientists studied the various lines of plant defense, focusing on preformed physical barriers, phytoalexins and other antimicrobial compounds that pre-existed or were formed post-infection (reviewed in Hückelhoven, 2007). However, with few exceptions, plant defense, pathogen virulence and host range (especially race-cultivar specificity) could not be adequately explained on such bases (reviewed in Hückelhoven, 2007) but instead was thought to rely largely upon induction of a series of host immune responses. Flor's seminal analysis of the complemen-

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tary genetic systems of the flax rust fungus (*Melampsora lini*) and its host, uncovered the complementary genetic systems of the pathogen and the plant, determining the outcome of race-cultivar interaction (either compatible or incompatible) and led him to formulate the “gene-for-gene hypothesis”, which explained in genetic terms the basis of race-cultivar specificity (Flor, 1971).

In the early 60s, Klement and his associates discovered that phytopathogenic bacteria, like fungi, were able to induce a rapid plant cell death at the infection site, also known as the plant hypersensitive response (HR), which was associated with restriction of pathogen proliferation (Klement and Goodman, 1967). Against this background, several parallel discoveries in the mid-1980s and the decade that followed led to the unified concept that bacterial virulence on plants relies heavily on sophisticated molecular machines, known as bacterial secretion systems or injectisomes, which enable them to target host defense systems at various levels. Key discoveries included the cloning of phyto-bacterial avirulence (*avr*) genes (Staskawicz *et al.*, 1984), the discovery of gene clusters including the *hrp* genes (phonetic “Harp”) (Panopoulos *et al.*, 1984; Panopoulos and Peet, 1985; Lindgren *et al.*, 1986), and the identification of virulence genes in animal pathogens (Isberg and Falkow, 1985). These were later shown to share homologies with *hrp* genes and to mediate secretion of virulence-related proteins lacking a canonical signal peptide (rev. in Tampakaki *et al.*, 2010). The boost in molecular functional genetics of bacteria that followed confirmed this concept and revealed the injectisomes’ organization and function. The latter is to help pathogens transfer (inject) directly into the plant cytosol proteins that affect immunity and are known as effector proteins. Today, the products of pathogen *avr* genes are collectively referred to as effectors, since in the absence of cognate plant immunity receptors they promote pathogen virulence by directly or indirectly interfering with plant defense mechanisms (Ritter and Dangl, 1996, Grant *et al.*, 2006; Jones and Dangl, 2006; Guo *et al.*, 2009). These findings

helped explain the enigma why phytopathogens carry avirulence genes (Dangl, 1994; Gabriel, 1999).

Plant immunity consists of different layers of defense, including a series of immune responses that are triggered post-infection by a variety of elicitors. One group of elicitors that are conserved among different bacterial pathogens includes the so-called pathogen-associated molecular patterns (PAMPs), which elicit PAMP-triggered immunity (PTI). Another group includes effectors, which as previously mentioned are often coded by classical avirulence genes. Molecular recognition of PAMPs by the host is mediated by proteins that recognize molecular structures conserved across a broad range of pathogenic species and are known as pattern recognition receptors (PPRs). Recognition of effectors on the other hand by the host is mediated by resistance proteins that are plasma membrane or intracellularly located.

It is now widely recognized that in their interactions with eukaryotes, Gram-negative bacteria use a variety of molecular devices, including extracellular appendages, to deliver diverse proteins and other molecules to the host cell interior (Charova *et al.*, 2012). With the rapid accumulation of bacterial genome sequences, our knowledge of the complexity of bacterial protein secretion systems has expanded and numerous biochemical studies have revealed the existence of at least six major mechanisms of protein secretion (Type I, II, III, IV, V and VI), which are often highly conserved among the Gram-negative bacteria species. This review focuses on the type III and type VI protein secretion systems (T3SS, T6SS) that mediate secretion and/or translocation of bacterial virulence-related proteins inside the host cell in a host contact dependent manner and under specific *in vitro* conditions (Hayes *et al.* 2010). Emphasis in this review is given on the biotechnological applications that emerged from the study of these systems, and range from plant pathogen diagnostics to antibiotic development. The review does not cover the Agrobacterial type IV secretion system, which has provided a basis for biotechnolog-

ical applications in the field of genetic engineering of crops on a commercial scale – the subject has been extensively reviewed elsewhere (Gelvin, 2010; Van Montagu, 2011).

T3SS function as vehicles of protein trafficking

T3SS are molecular nanomachines found in many Gram-negative proteobacteria (α , β , γ and δ subdivisions, also found in some non-proteobacteria such as *Chlamydiae*) that establish intimate relationships (pathogenic, symbiotic or commensal) with plant, human, insect, nematode or animal hosts and are evolutionarily related to bacterial flagella (Dale and Moran, 2006; Tampakaki *et al.*, 2004; Troisfontaines and Cornelis, 2005; Charova *et al.*, 2012). They function as “injectisomes”, translocating diverse repertoires of proteins to extracellular locations and into eukaryotic cells. Many T3SS-secreted proteins (T3SS effectors) modulate the function of host regulatory networks, which determine the outcome of recognition and biological accommodation with the eukaryotic host. T3SS have evolved into seven phylogenetic families (Troisfontaines and Cornelis, 2005). Some bacteria may harbor more than one T3SS, which usually belong to different phylogenetic families (non-orthologous), an indication of relatively recent horizontal gene transfer.

In plant pathogenic bacteria, the T3SS forms a long pilus that equals or exceeds in length that of the bacterial cell and has a central channel through which the secreted proteins are transported from the prokaryotic cytoplasm across the inner and outer bacterial membrane as well as the plant cell wall, into the host cytosol. The proteins that build and regulate the assembly and function of this secretion apparatus are coded by three classes of genes (reviewed in Tampakaki *et al.*, 2010). Firstly, the *hrc* genes (*hrp* conserved), which also have homologs in the bacterial flagellum and whose protein products are conserved at the sequence level among animal and plant pathogenic bacteria. They express proteins known as the T3SS core components, including HrcJ, HrcU,

HrcV, HrcN, HrcR, HrcT, HrcS and HrcC, which form the basal body of the apparatus that is embedded in the inner and outer bacterial membrane. HrcC forms a ring-like structure in the outer membrane. HrcN and its homologs catalyze ATP hydrolysis providing energy to the system and are involved in protein unfolding and transport (Lorenz and Büttner, 2009). Secondly, transcriptional regulators of the T3SS operons and effector genes, such as a) the alternative sigma factor (σ^I) HrpL, the master regulator of the secretion system in *P. syringae* pathovars, *Pantoea*, *Pectobacterium* and *Erwinia/Dickeya* species, and b) the AraC-type regulators HrpB and HrpX in *Ralstonia* and *Xanthomonas* species, respectively (Büttner and Bonas U., 2009). Importantly, *avr*/effector genes and *hrp/hrc* operons are co-regulated, a finding that provided an early clue about their functional linkage. The third class of genes codes for the extracellular T3SS components (the pilus subunits, HrpA in *P. syringae* and *Erwinia amylovora*, HrpY in *R. solanacearum*, HrpE in *X. campestris*), proteins serving as chaperones assisting in injectisome assembly, secreted proteins with extracellular enzymatic function, proteins forming pores in the plant cell wall or assisting translocation activity (HrpK, HrpF; reviewed in Büttner and He, 2009) and proteins such as harpins (HrpZ, HrpW, PopA; He *et al.*, 1993) that are able to elicit the HR in purified form.

The *hrp/hrc* genes are generally located in pathogenicity islands (PAIs) and are primarily found on the bacterial chromosome rather than on plasmids. Typical *hrp* clusters, such as those of *P. syringae* pathovars, consist of core regions of 6 operons and 27 open reading frames (ORFs), flanked by two effector rich loci, the conserved effector locus (CEL) and the exchangeable effector locus (EEL; Alfano *et al.*, 2000). New *in silico* analysis revealed multiple T3SS clusters in the same strain, found not to be non-orthologous and functionally non-redundant but serving bacterial virulence in different ways. For example, in case of *E. amylovora*, one T3SS mediates interaction with the plant host, while the second might be serv-

ing dissemination by insect vectors (Triplett *et al.*, 2006).

The type VI secretion system

The recently discovered T6SS is typically encoded by clusters of 13 genes thought to constitute the minimal set needed to produce a functional secretion apparatus. Our current knowledge concerning T6SS regulation of gene expression and apparatus components has been obtained basically from studies on human and animal bacterial pathogens (Boyer *et al.*, 2009). T6SS occur mostly in α -, β -, and γ -proteobacteria (about 25% of the sequenced genomes; Bingle *et al.*, 2008) and in *Helicobacter hepaticus* (Chow and Mazmanian, 2010), a member of the ϵ -(ϵ -epsilon) group, which is an intestinal microbiote. Multiple copies of these clusters (often referred to as T6SS loci in the literature) are frequently found in the same genome and are non-orthologous, indicating that they did not originate from internal duplication in the bacterial host but were probably acquired independently by horizontal transfer (Sarris *et al.*, 2010). In few such cases that have been studied, each T6SS assumes a different role in the interactions of the bacterial organism with others.

At present, only few T6SS substrates have been verified experimentally, but others may merely await identification. Some of the T6SS core component proteins are structurally related to the cell-puncturing devices of tailed bacteriophages, and at least in some well-studied cases, the system has been shown capable of translocating effector proteins into the host cell cytoplasm, and thus is implicated in virulence of certain plant pathogenic bacteria, including *Agrobacterium tumefaciens*, *Pectobacterium atrosepticum* and *Xanthomonas oryzae*, as well as in the multi-host pathogen *Pseudomonas aeruginosa* strain PA14 (reviewed in Sarris *et al.*, 2012). However, the T6SS is not only implicated in pathogenesis, as several reports attribute a fundamental role in efficient root colonization/nodule formation by the nitrogen-fixing plant symbionts/mutualists *Mesorhizobium loti*, *Rhizo-*

bium leguminosarum and *Cupriavidus taiwanensis* (Bingle *et al.*, 2008).

The diverse role of Bacterial effectors—not the last words

Bacterial T3SS effectors constitute a large and diverse group of virulence proteins with a wide range of cellular-subcellular targets and biochemical functions. A prominent feature of these proteins is their modular architecture, comprised by domains or motifs that confer an array of biochemical functions within the eukaryotic cell. They are in essence molecular chimeras of functionally distinct sequence domains, distributed in different ways along the effector sequence properly but with some common rules. For example, sequence motifs required to direct secretion from the bacterial cell, translocation into the host cell and membrane localization, are generally located to the N-terminus of the protein, while functional domains and motifs mediating subcellular targeting and interactions with host targets typically localize to the central or C-terminal portions. This modularity has been exploited for construction of reporters to monitor secretion/translocation and in genetic screens designed to identify new effectors. An impressive variety of structural motifs are found in various combinations in T3SS effectors (Dean, 2012), which may be viewed as “genetic jugglery” to borrow a term from Davis and Davis (2010).

The motifs required for T3SS are located in the first 15–25 N-terminal residues, but without discernible consensus, and a chaperone binding domain 50 to 150 residues downstream. Subcellular targeting signals include mitochondrial targeting motifs, membrane targeting motifs nuclear localization signals, caspase processing sites and G-protein regulatory domains, a variety of motifs involved in protein–protein interactions, and a long list of catalytic activities (Dean, 2012). These features form the bases of the diverse cellular roles of effectors in bacteria–host interactions.

Particular effectors have been found to control bacterial proliferation on the plant

surface, prevent stomata closure and inhibit enzymatic degradation of bacterial peptidoglycans in the leaf apoplast. Moreover, they mask elicitor recognition and block signaling receptors. They are able to down-regulate, suppress and/or degrade defense mechanisms such as callose deposition and papillae formation, kinase phosphorylation and dephosphorylation, resistance protein activation, and block the hypersensitive response triggered by other effectors (reviewed in Jones and Dangl, 2006; Göhre and Robatzek, 2008). Moreover, they modulate plant transcription, proteasomal degradation machinery and hormone-signaling networks (involved in defense signaling) by down regulation of microRNAs in the RNA silencing mechanism (Navarro *et al.*, 2008; Zhang *et al.*, 2011). The plant defense-suppression functions of *P. syringae* effectors are summarized in Table 1.

Large scale genome sequencing and subsequent effector prediction, answered the riddle of effector redundancy and revealed the rich effector repertoire of different pathogens: Studies by Sharkar *et al.* (2006) on host specificity showed that low conservation of T3SS effector repertoires among different bacterial pathovars and species underlies their differences on host specificity, while high conservation of T3SS effectors can explain pathogenicity and a broad host range. Further on, acquisition of new T3SS effectors by HGT or other genetic mechanisms can widen a pathogen's host range (Jones and Dangl, 2006). It is therefore evident that the effector repertoire of a pathogen largely determines its infection strategy as well as its host range.

Biotechnological applications

Mining for effectors reveals new potential tools in plant breeding

Following Flor's hypothesis and using Klement's technique (see introduction), different panels of scientists determined that plant resistance was associated with HR elicitation to specific pathogen races. This led

to the characterization of many avirulence (effector) genes and their matching plant resistance (*R*) genes, which in some cases enabled the full characterization of resistance due to *avr-R* interacting gene pairs in several pathosystems at the race/cultivar or species level. One of the first resistance genes to be cloned was *Bs1* from pepper accession PI163192, which interacted with the protein effector encoded by the *avr* gene *avrBs1* from *X. campestris* pv. *vesicatoria* (Minsavage *et al.*, 1990). Backcross programs were initiated to transfer the resistance gene into commercially valuable cultivars (Early Calwonder). Similar efforts will help the development and/or identification of several cultivars of pepper (*Bs1-4*) and tomato (*rx1-3*, *Xv3*, *Xv4*) with resistance towards bacterial spots caused by *Xanthomonas* spp. (reviewed in Stall *et al.*, 2009). Indeed, several pepper varieties resistant to three or more of the six bacterial leaf spot races are now commercially available (Sweet Bell, Sweet Italian, Hot peppers etc.). Also in the early nineties, Martin *et al.* (1991) isolated the *Pto* kinase *R* gene, which confers resistance to bacterial speck disease in tomato by recognition of the corresponding *avrPto* avirulence gene, in the pathogen *P. syringae* pv. *tomato*. Similarly, breeding efforts with rice *R* genes such as *Xa1-26* that confer resistance to bacterial blast caused by *Xanthomonas oryzae* pathovars, are now implemented in control measures along with cultural practices, chemical and biological control, and disease forecasting (Niño-Liu *et al.*, 2006). Several other efforts have been reported on different pathosystems. Genome sequencing programs and protein motif comparison and function prediction analysis facilitate similar efforts and will provide breeders and geneticists with a significant number of resistance genes in the near future.

TAL Effectors: Novel tools for gene targeting and genome engineering

Transcription activator-like effectors (TALEs) are T3SS-secreted avirulence proteins found in *Xanthomonas*. These proteins can bind promoter sequences in the host plant

Table 1. Biochemical functions of Type III secretion system effector proteins of *Pseudomonas syringae* bacteria and role in HR or/and the plant innate immunity mechanisms.

Effector	Function	Ref. strain	Arbidopsis			Yeast*			<i>N. benthamiana</i> ^b			Avirulence		
HopAB2 (avrPtoB)	Suppresses cell death & HR triggered by other effectors, suppress FRK1/SIRK alters ethylene responses; targets Pto/Fen/PAMP & RLKs/BAK1	<i>P.s.t</i> DC3000	v	v	v							Tomato		
HopX1 (avrPphE_{pro})	Induces JA -responsive genes; suppress HopA1-dep. HR & RAP2.6	<i>P.s.t</i> DC3000	v	v	v							Pea/Bean		
HopAM1 (avrPpiB_{pro})	Enhances virulence on water-stressed plants	<i>P.s.t</i> DC3000	v	v	x							Tomato		
HopD1 (HopPtoD1)	Intermediate ability to suppress HR	<i>P.s.t</i> DC3000	v									Tomato		
HopE (HopPtoE)	Inhibits HR & Arabidopsis WNK Kinase	<i>P.s.t</i> DC3000	v	v	v							Lettuce		
HopF2 (HopPtoF)	Suppressing the flagellin-induced <i>NHO1</i> expression; targets R proteins RIN4/MKK5	<i>P.s.t</i> DC3000	v	v	v	v	v	v						
HopF1 (avrPphF)	Suppresses R-gene induced cell death	<i>P.s.ph</i> 1449B										v	Bean	
HopK1 (HopPtoK)	Intermediate ability to suppress HR; suppress RAP2.6	<i>P.s.t</i> DC3000	v	x									Lettuce	
HopG1 (HopPtoG)	Targets mitochondria, alters plant development & suppresses innate immunity	<i>P.s.t</i> DC3000	x	v	v	v	v	x					Tomato	
HopAO1	Suppresses cell death Induce JA-resp. genes	<i>P.s.t</i> DC3000	v ^a											
AvrRps4	Suppression of PTI	<i>P.s.pi</i> 151	v										Arabidopsis	
AvrPto1	Suppressing the flagellin-induced <i>NHO1</i> & FRK1/SIRK; interacts with Rab-GTPases; targets Pto/Fen/PAMP	<i>P.s.t</i> JL1065	x	v	v	v	v	v					v	Tomato
AvrB1	Suppresses basal defences & RAP2.6; Induce JA-responsive genes targets R prot. RIN4/RAR1/MPK4	<i>P.s.gl</i> R4 & R0											v	Soybean

HopM1 (HopPtoM)	Degradation of AtMIN7 to suppress PTI & SA-regulated immunity; targets MIN7 and others	<i>P.s.t</i> DC3000	v	v	x	Tomato
AvrE1	Suppress defences by perturbing host's G-protein regulation	<i>P.s.t</i> DC3000	v	v	x	Arabidopsis
HopC1 (HopPtoC)	Inhibit flagellin-induced NHO1 expression	<i>P.s.t</i> DC3000	x	x	x	Lettuce
HopN1 (HopPtoN)	Is localized to chloroplasts, degrades PsbQ & inhibits PSII	<i>P.s.t</i> DC3000	v			
HopAB1 (VirPphA)	Suppression of HR	<i>P.s.ph</i> R7 1449B	v	v	v	
AvrB2 (avrPphC)	Suppress cell death triggered by other effectors	<i>P.s.ph</i> R7 1449B				Bean, Pepper
HopZ3 (HopPsyV)	Suppress isoflavone biosynthesis	<i>P.s.s</i> B728a	v			Cotton, Lettuce, Tomato, Pepper, <i>N. benthamiana</i>
AvrRpt2	Suppresses basal defences, RPM1-induced HR; suppresses RAP2.6; targets RIN4	<i>P.s.t</i> JL 1065	v			Arabidopsis, Pepper, Tomato
AvrRpm1	Suppresses basal defences; target RIN4	<i>P.s.ma</i> M6	v			Soybean, Arabidopsis
HopZ2 (avrPpiG1)	Targets host <i>SUMOylated</i> proteins; Degradation of PBS1	<i>P.s.pi</i> 151			v	
HopART (avrPphB)	Induces JA -responsive genes Degradation of PBS1; Suppresses RAP2.6; targets PBS1/BIK1/PBLs	<i>P.s.ph</i> R4 & R3	v			Bean, Arabidopsis Tomato, Cotton, Lettuce
HopAI1 (HolPtoAI)	Targets MAPK-signalling & flagellin-induced NHO1 expr.	<i>P.s.t</i> DC3000	v			Lettuce
HopS1 (HolPtoZ)	Suppressing the flagellin-induced <i>NHO1</i> expression	<i>P.s.t</i> DC3000	v			
HopAF1 (HopPtoJ)	Suppressing the flagellin-induced <i>NHO1</i> expression	<i>P.s.t</i> DC3000	v			
HopT1-1 (HopPtoT1)	Suppressing the flagellin-induced <i>NHO1</i> expression	<i>P.s.t</i> DC3000	v			Arabidopsis, Tomato, Lettuce
HopT1-2 (HolPtoU2)	Suppressing the flagellin-induced <i>NHO1</i> expression	<i>P.s.t</i> DC3000	v			
HopAA1 (HopPtoA1)	Suppressing the flagellin-induced <i>NHO1</i> expression	<i>P.s.t</i> DC3000	v			<i>N. benthamiana</i> , Tomato, Lettuce, Cotton
HopI1	Causes chloroplast thylakoid structure remodelling and suppresses SA accumulation; targets Hsp70	<i>P.c.c.al</i> ES4326	v			
HopU1	Suppresses plant innate immunity has mono-ADP-ribosyltransferase activity; targets GRP7 and other	<i>P.s.t</i> DC3000	v	v		

Effector nomenclature in parenthesis refers to their original names. Table depicts effector suppression function in different hosts tested, original avirulence phenotypes to certain host cultivars (mentioned first) and new avirulence functions found. Abbreviations mentioned in effector function correspond to plant resistance proteins and proteins related to plant defense signaling. HR: Hypersensitive response. PAMP: pathogen-associated molecular pattern. JA: Jasmonic acid. P.s.: *Pseudomonas syringae* pathovars; t: tomato; ph: *phaseolicola*; ma: *marulicola*; pi: *pisu gl. glycinea*; P.c.al.: *Pseudomonas cannabina* pv. *alisalsensis*.

v: Suppression function

x: No suppression function

*: Ability to suppress Bax-induced killing of Yeast

- a: HopAO1 in Arabidopsis suppresses callose deposition elicited by the Pst DC3000 hrpA mutant, and allows the normally non-pathogenic hrpA mutant to multiply within the leaf tissue. HopAO1 also suppresses resistance to Pst DC3000 induced by flg22, a PAMP. However, HopAO1 does not suppress the HR triggered by several classical avirulence genes
- b: I. Challenge HR electrolyte leakage, II. Vascular dye accumulation, III. Suppression of hypersensitive response (HR)

Table Adapted from:

1. *Mol. Plant Microb. Interact.*, 17: 1328–1336, 2004. *Pseudomonas* Type III Effector AvrPto Suppresses the Programmed Cell Death Induced by Two Non-host Pathogens in *Nicotiana benthamiana* and Tomato.
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and activate the expression of plant genes that aid in bacterial infection. They recognize plant DNA sequences through a central domain consisting of a variable number of tandem, 33–35 amino acid repeats, followed by a single truncated repeat of 20 amino acids. The native function of these proteins is to directly modulate host gene expression. Upon delivery into host cells via the bacterial T3SS, TAL effectors enter the nucleus, bind to effector-specific sequences in host gene promoters and activate transcription (Boch and Bonas, 2010; Voytas and Bogdanove, 2011). The DNA binding code of TAL effectors is fairly simple: a hypervariable pair of adjacent residues at positions 12 and 13 in each repeat, the 'repeat-variable di-residue' (RVD), specifies the target, one RVD to one nucleotide, with the four most common RVDs each preferentially associating with one of the four bases in the DNA target. In naturally occurring TAL proteins the recognition sites are uniformly preceded by a T nucleotide that is required for TAL effector activity (Voytas and Joung, 2009; Moscou and Bogdanove, 2009; Boch *et al.*, 2009).

These straightforward sequence relationships between the variable amino acids in TAL effector repeats and DNA bases in their target sites both allow the prediction of TALEs and their target sites and enable the redesigning of effectors to selectively bind to DNA targets of choice. These proteins are interesting to researchers both for their role in disease of important crop species and the relative ease of retargeting them to bind at pre-chosen DNA sequences. Similar proteins can be found in the pathogen *Ralstonia solanacearum*.

Genome sequencing generates opportunities to strategically manipulate selected genes in DNA targeting for a wide range of applications: understanding gene function in model organisms, reprogramming the regulation of selected loci in higher eukaryotic genomes through novel transcription factors, site directed mutagenesis and other techniques. Potential applications are envisioned in treating human genetic disorders, improving traits in crop plants, genome en-

gineering and synthetic biology. At present, a serious technical limitation of such applications is the difficulty of altering nucleotide sequences and expression of genes in living cells in a targeted fashion. The TAL effectors of plant pathogenic *Xanthomonas* have provided researchers with a new tool to meet this challenge. Fusions of these effectors to rare-cutting restriction endonucleases (called TALENs, TALE nucleases), or other programmable nucleases, to customizable arrays of polymorphic amino acid repeats, direct the nuclease to particular DNA sites which they subsequently cleave (Bogdanove and Voytas, 2011; Morbitzer *et al.*, 2010). In a recent plant-related application (Li *et al.*, 2012) a TAL effector of *Xanthomonas oryzae* pv. *oryzae* (Xoo), which transcriptionally activates a specific rice disease-susceptibility (*S*) gene, was fused to the DNA cleavage domain of *FokI* to create a TALEN able to edit the particular *S* gene, altering the susceptibility response and thereby engineering resistance to bacterial blight.

Harpins

T3SS proteins of phytopathogenic bacteria, such as harpins, have been studied for their use in crop protection. The first example was the use of the harpin protein HrpN from *E. amylovora* overproduced in a heterologous bacterial system (*Escherichia coli*) (Wei *et al.*, 1992). Harpins elicit a complex set of metabolic responses in the treated plant, which result in promotion of plant growth, induction of defense responses against different types of pathogens and insects, and tolerance to drought stress (reviewed in He *et al.*, 2004). Harpins or harpin-like proteins such as HrpZ from *P. syringae* pv. *syringae* (Strobel *et al.*, 1996), PopA from *Ralstonia solanacearum* (Belbahri *et al.*, 2001) also elicit similar reactions on non-host plants. Transgenic cotton lines expressing Harpin_{Xoo} from *X. oryzae* pv. *oryzae* accumulate harpin along the plant cell wall where it likely acts as an endogenous elicitor (Miao *et al.*, 2010), leading the plant to a primed state which improves resistance against *Verticillium* wilt. Similarly, transgenically expressed

HrpZ protein fused to a plant signal peptide enhanced “rhizomania” resistance both in a model plant (*Nicotiana benthamiana*) and sugar beet, the natural host of BNYVV (Pavli *et al.*, 2011). However, the exact action site of harpins remains unknown. Tampakaki and Panopoulos (2000) suggested that the harpin receptor could be extracellular in transgenic tobacco expressing HrpZ of *P. syringae* pv. *phaseolicola*, while other groups proposed the plasma membrane as the location of harpin receptor using immune-cytological experiments (Lee *et al.*, 2001).

Harpin’s potential culminated from basic and applied research that led to the registration and placing in the market plant protection products with various harpins as the active ingredients, by companies such as EDEN Bioscience_{TM}, which launched MessengerTM and ProActTM as biopesticides for yield enhancement and disease management. This novel crop management technology offers the advantages of being environmentally friendly as it does not leach, bioaccumulate, or persist in the environment, and leaves no detectable residues on treated crops. Nevertheless, its small share in the global market indicates that there are limitations to this technology and probably space for improvement.

Hrp-based Diagnostics

Conventional methods for detection of bacterial plant pathogens relies on culture-based methods, biochemical and serological typing (immunofluorescence, enzyme-linked immunosorbent assay - ELISA), protein profiling (SDS-PAGE), fatty acid methyl-ester (FAME) profiling and pathogenicity confirmation testing. However, detection is increasingly depending on polymerase chain reaction (PCR) - based techniques, which mainly focus on amplification of the 16S rDNA gene and the 16S-23S internal transcribed spacer by genera/species specific primers, combined occasionally with simple restriction fragment length polymorphisms (RFLPs) and repetitive-sequence-based PCR (REP-PCR) analysis. A list of internationally agreed diagnostic proto-

cols for regulated pests is published by the European and Mediterranean Plant Protection Organization (EPPO, 2012). Other primer target genes usually include genes involved in basic cellular functions, as for example the *Rhs* gene family (Park *et al.*, 2006; Albuquerque *et al.*, 2012) and gyrase B (*gyrB*) (Weller *et al.*, 2007). Moreover, several specific primers have been designed on genera and species specific genes, such as the genes controlling production of bacterial phytotoxins, such as phaseolotoxin or coronatine for the detection of the bean halo blight (Schaad *et al.*, 1995) and the bacterial speck pathogen of tomato (Cuppels, 2006). The main drawbacks so far of PCR-based methods are sensitivity, cross reaction with other bacteria, and false negatives/positives, usually due to the DNA extraction method and/or the plant tissue. Real time, multiplex and competitive PCR protocols have been developed to overcome such limitations (Pastrik, 2000; Berg *et al.*, 2006; Chen *et al.*, 2010).

The use of primers targeting functional systems that only pathogenic bacteria possess, such as the T3SS, could offer an alternative tool to overcome the aforementioned limitations. In this direction, there have been reports for the detection of Gram-negative pathogens by PCR amplification of *hrc* genes by means of genus, species or pathovar specific primers. In their pioneering work, at a time when full annotations of the *hrp* cluster were not available, the Stall and Bonas group (Leite *et al.*, 1994) had foreseen the potential of *hrp* genes for diagnostic purposes. They have used genomic DNA of a *X. campestris* pv. *vesicatoria* strain and DNA from plasmids that contained cloned parts of the *hrp* cluster to develop oligonucleotide primers specific for different *hrp* regions, which amplified DNA from *X. fragariae* and 28 pathovars of *X. campestris*. More recently, other groups have also used the same set of primers for *Xanthomonas* species either *per se* or as a basis for designing new primer sets against additional *Xanthomonas* species and pathovars, based on the load of available sequences. Indeed, Obradovich *et al.* (2004) differentiated the *X. campestris* pv.

vesicatoria groups in isolations from pepper and tomato with the same primers as Leite *et al.* (1994), while Park *et al.* (2010) designed new primers that specifically amplified a 243-bp product from genomic DNA of *X. arboricola* pv. *pruni* strains, and not from the 21 other strains of *Xanthomonas* and two epiphytic bacterial species. In addition, Zaccardelli *et al.* (2007) proposed a rapid detection method for *X. campestris* pv. *campestris* in crucifer seeds and plants, based on amplification under stringent conditions of an internal *hrcC* fragment of 519 bp from a number of isolates and by means of pathogen-specific primers.

Recently, conventional PCR assays were adapted to real-time PCR to develop more sensitive and rapid techniques. Even if most of these studies target gene sequences other than those involved in T3SS function, as for example those coding for putative members of the ATP-binding cassette (ABC) transporter family in *X. arboricola* pv. *pruni* (Palacio-Bielsa *et al.*, 2011), there have been pilot reports on real-time PCR diagnostic assays based on *hrp* genes. Berg *et al.* (2006) for example, used fluorescently labeled probes to develop a multiplex PCR amplifying a 78-bp segment of the *hrpF* gene from different *X. campestris* pathovars and a 100-bp segment of the *Brassica* spp. 18S–25S internal transcribed spacer. The latter provided an internal control for the amplification process to prevent false negatives. Nevertheless, several real-time PCR protocols have been published in the last few years based on T3SS gene sequences of mammalian pathogens. Most of these PCR protocols target gene sequences such as *hilA* and *ssaN*, a T3SS transcriptional regulator and a putative T3SS ATP synthase gene of *Salmonella enterica* serovars, respectively, of pathogens borne in food and dairy products (McCabe *et al.*, 2011; Chen *et al.*, 2010). Moreover, similar assays target genes coding for T3SS secreted effectors such as *tccP* and *tccP2* from pathogenic *Escherichia coli* (Madic *et al.*, 2011). Considering the fact that both basic and applied research on bacterial secretion system progress since the very beginning hand in

hand for plant and mammalian pathogens, one should only expect that use of real time PCR with sensitive and efficient probes (such as the TaqMan technology), will also expand rapidly in the next few years for phytopathogen detection. This approach offers the advantage that detection can be completed within a few hours with a sensitivity of 10^2 CFU ml⁻¹, thus surpassing the sensitivity of the existing conventional PCR.

The microarray technology offers a promising tool for transcriptional profiling, which has also been exploited for T3SS expression profiling and regulation (Ferreira *et al.*, 2006). Moreover, microarrays have recently been exploited for microbial species detection, community profiling on specific niches and/or hosts. High density microarrays for example, based on gene sequences encoding mainly for the small ribosomal subunit (16S) rRNA, have been developed for microbe/pathogen monitoring (Brodie *et al.*, 2007). Such tools could complement the currently available methods for pathogen detection, which are time consuming, and specific, but still lack economic feasibility. The approval of DNA microarray technology by governmental authorities (US Food and Drug Administration) has paved the way for new applications to clinical diagnostics and pathogen detection in foods and crops.

Microarray applications for the detection of plant pathogens have already been developed, mainly targeting a specific host plant. Probe designing on most cases has been based on the 16S rRNA gene region with the complementation of pathogenicity islands containing known virulence genes (Aittamaa *et al.* 2008) or housekeeping genes such as *rpoB*, *groEL*, *ftsZ* (Pelludat *et al.*, 2009) and *gyrB* (Kostic *et al.*, 2007). The diagnostic ability could extend from genus and species to pathovar level. However, such diagnostic tools are still in their infancy with plant pathogens as they have been applied to pure culture lysates rather than infected plants and cross hybridization between probes often occurs. The use of *hrp* genes could address the latter problem, as although they are conserved, they still in-

clude regions of high polymorphism. Based on pilot studies from the authors of this manuscript (Skandalis *et al.*, 2010), we suggest that microarray target probe designing on *hrc* genes could be a valuable tool for detection of a wide range of Gram-negative plant pathogens as well as for simultaneous infections. In support, Garrido *et al.* (2006) already developed a diagnostic oligonucleotide array for the detection of shiga toxin-producing and enteropathogenic *E. coli* strains based on the genetic variability present at the enterocyte effacement (LEE) pathogenicity locus, which contains genes encoding for the T3SS and T3SS effector proteins.

Are secretion systems the key to a new generation of antibiotics?

Development of resistance to antibiotics that target bacterial viability, urged for new antimicrobial development strategies that target various pathways related to virulence, including toxin function, toxin delivery, regulation of virulence expression and bacterial adhesion (reviewed in Clatworthy *et al.*, 2007). Inhibition of these virulence factors would render hazardous bacteria non-infectious only within the host, thereby reducing the selective pressure for resistance development, and would not perturb the nontarget microflora. In this regard, T3SS apparatus components that are linked to the outer membrane or exposed, offer an attractive substrate for inhibitor (antibiotic) development. Hence, screening for T3SS inhibitors has involved four distinct approaches, yielding inhibitors of T3SS transcription, assembly and specific effector protein secretion (reviewed in Lee and Kessler, 2009). In particular, small molecules termed Innate Pharmaceuticals AB (INPs), and in particular acylated hydrazones of different salicylaldehydes were found to prevent molecule translocation for *Yersinia pseudotuberculosis* (Nordfelth *et al.*, 2005), inhibit intracellular growth but not invasion of *Chlamydia trachomatis* into host cells (Muschiol *et al.*, 2009), inhibit the T3SS and virulence of *Salmonella enterica* serovar *Typhimurium* (Hud-

son *et al.*, 2007) and finally inhibit secretion assembly of *Shigella flexneri* T3SS. Effector protein inhibitors were identified either –in the case of pseudolipase A – on the basis of inhibition of host cell lysis by the *P. aeruginosa* ExoU cytotoxin effector or alternatively, by screening for compounds, such as exosin and 0433YC1-2, that permitted the growth of yeast with induced expression of cytotoxic effectors of *P. aeruginosa* (ExoS) and *Chlamydia pneumoniae* (CopN). Determination of 3-D structures of T3SS components and effectors provide unexplored options for designing antivirulence chemicals, including next generation virulence inhibitors binding to adjacent, functionally relevant binding pockets of T3SS components to reduce the chances of resistance development.

Conclusions

Historically, the genes coding for the T3SS were identified in phenotypic screens of mutants altered in their interaction with other organisms (higher eukaryotes). The identification of effectors in plant pathogens also had a similar starting point (transfer of whole genome libraries from avirulent to virulent strains and carrying out HR screens on *R* gene differentials; Staskawicz *et al.*, 1984; Table 1). The discovery of the T6SS followed the opposite route: its existence emerged from bioinformatic sourcing of genomic data. It is conceivable that future sourcing of genomic, proteomic, transcriptome and structural database data may point to new potential candidates, particularly for effectors. Possibly, new secretion systems and new effectors may even be predicted, “the way Mendeleev had anticipated characteristics of yet unknown elements”, as is already the case with TAL effector DNA binding specificities.

Large scale analysis of interactions of effectors with different hosts by means of *Agrobacterium* transient expression (Wroblewski *et al.*, 2009; Lindeberg *et al.*, 2012) confirmed that their suppression and avirulence function varied significantly, espe-

cially among distant plant families, some of which are highlighted in Table 1. This might reveal new potential in genetic engineering towards phytoprotection, as exploitation of a certain effector function in a specific crop could lead to priming of defense responses upon challenge with a pathogen. Moreover, the genomics era for plant pathogens, which began with the sequencing of *Xylella fastidiosa* genome in year 2000 (Simpson *et al.*, 2000) and the new comparative analysis of genomes (Hamilton *et al.*, 2011), which allows for identification of conserved and divergent features, thus leading to species, pathovar and isolate-specific genes, supports diagnostics research towards new molecular kits based on such targets, where both *hrp* genes and T3SS effectors might be in the centerfold.

With regard to applications, it is perhaps significant that various uses of bacterial effectors have been the subject of several patents, to enable possible applications in a number of areas, including plant disease and pest control. In addition, new frontier areas are likely to emerge from the developments in high-throughput DNA sequencing (next-generation sequencing technologies). Very high-throughput DNA sequencing platforms are commercially available at affordable costs and new more cost effective techniques are under development. Rapid genome-wide characterization and targeted capture of genomic subsets and polymorphisms from many phytopathogen strains at a time, and public access to genome, transcriptome and metagenome data will likely reshape many areas of "phytopathogenics" and will enable applications in disease diagnosis, epidemiology, effectoromics and comparative pathogenomics and the development of resistance strategies in the future. Characteristic of this trend are two recent publications (Baltrus *et al.*, 2012; Bart *et al.*, 2012), which concern the sequencing of new 14 and 65 phyto bacterial genomes, respectively.

Research on antibacterial compounds for phytoprotection could follow the mammalian research initiatives. Specificity of

T3SS virulence inhibitors could render field application feasible, in contrast to conventional antibiotics. However, development of such molecules would face drawbacks similar to those of the mammalian pathogens: post-infection usage, broad range activity, targeting of infected tissue and non-target organism safety issues. In addition, the lack of an adaptive immune system would render T3SS therapeutics more complicated and disqualify methods such as usage of secreted effectors as vaccines and T3SS mutants as live vaccines, which are under investigation for mammalian bacterial pathogens (see Coburn *et al.*, 2007).

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ΑΡΘΡΟ ΑΝΑΣΚΟΠΗΣΗΣ

Τριάντα χρόνια έρευνας στα εκκριτικά συστήματα των βακτηρίων: Βιοτεχνολογικές εφαρμογές και οφέλη στη φυτοπροστασία

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Περίληψη Η ανακάλυψη στα μέσα της δεκαετίας του '80 ενός εκκριτικού συστήματος πρωτεϊνών, που ονομάστηκε εκκριτικό σύστημα τύπου III (T3SS), κοινού σε παθογόνα φυτών και ζώων καθώς και σε συμβιωτικά βακτήρια, αποτέλεσε ορόσημο στην φυτοπαθολογία και συγκεκριμένα στις αλληλεπιδράσεις φυτών-βακτηρίων. Τα φυτοπαθογόνα βακτήρια πρέπει να καταβάλουν τα πολλαπλά επίπεδα άμυνας του φυτού που είτε προϋπάρχουν είτε επάγονται μετά τη μόλυνση. Για να το επιτύχουν, χρησιμοποιούν σύμπλοκα πρωτεϊνών που δημιουργούν δομές κατάλληλες για την έκκριση πρωτεϊνών τόσο στον εξωκυτταρικό χώρο του βακτηρίου, όσο και απευθείας στο κύτταρο του φυτού ξενιστή. Οι πρωτεΐνες αυτές εμποδίζουν την αναγνώριση των παθογόνων από τον ξενιστή, αποτρέπουν την επαγωγή αμυντικών μηχανισμών και παρεμβαίνουν στην σηματοδότηση που οδηγεί στην ενεργοποίησή τους. Πρόσφατα επιτεύγματα στη μοριακή βιολογία βοήθησαν την έρευνα στον τομέα των αλληλεπιδράσεων και έχουν καταστήσει τη μεταφορά πρωτεϊνών με το T3SS ως θεμελιώδη στην παθογένεια, τη μόλυσματικότητα και τον καθορισμό του εύρους των ξενιστών των gram- φυτοπαθογόνων βακτηρίων. Σε αντίθεση με τη πρόοδο στη γνώση μας στη βιολογία των παθογόνων, λίγα πράγματα έχουν αλλάξει στις μεθόδους και τον τρόπο καταπολέμησής τους. Στην παρούσα ανασκόπηση παρουσιάζεται συνοπτικά η λειτουργία και ο τρόπος δράσης του T3SS. Η έμφαση δίνεται στις βιοτεχνολογικές εφαρμογές που έχουν προκύψει με άξονα τη βασική έρευνα στο T3SS, από την ανάπτυξη νέων διαγνωστικών μεθόδων και την βελτίωση της ανθεκτικότητας των φυτών σε παθογόνα, στη δημιουργία αντιβιοτικών και τεχνολογιών για γονιδιακή στόχευση στα φυτά.

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