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Microreview

Recent developments in effector biology of filamentous plant pathogens

Ricardo Oliva,[1] Joe Win,[1] Sylvain Raffaele,[1] Laurence Boutemy,[2] Tolga O. Bozkurt,[1] Angela Chaparro-Garcia,[1] Maria Eugenia Segretin,[1] Remco Stam,[1] Sebastian Schornack,[1] Liliana M. Cano,[1] Mireille van Damme,[1] Edgar Huitema,[3] Marco Thines,[1,4] Mark J. Banfield[2] and Sophien Kamoun[1]*

[1]*The Sainsbury Laboratory, Norwich NR4 7UH, UK.*
[2]*Department of Biological Chemistry, John Innes Centre, Norwich NR4 7UH, UK.*
[3]*Division of Plant Sciences, College of Life Sciences, University of Dundee at Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK.*
[4]*University of Hohenheim, Institute of Botany 210, 70593 Stuttgart, Germany.*

Summary

Filamentous pathogens, such as plant pathogenic fungi and oomycetes, secrete an arsenal of effector molecules that modulate host innate immunity and enable parasitic infection. It is now well accepted that these effectors are key pathogenicity determinants that enable parasitic infection. In this review, we report on the most interesting features of a representative set of filamentous pathogen effectors and highlight recent findings. We also list and describe all the linear motifs reported to date in filamentous pathogen effector proteins. Some of these motifs appear to define domains that mediate translocation inside host cells.

Introduction

Plant pathogenic fungi and oomycetes, here referred to as filamentous pathogens, cause a variety of disease pathologies in natural and agricultural plant communities. Filamentous pathogens are intimately associated with their host plants and are sophisticated manipulators of plant cells. They achieve this by secreting an arsenal of molecules, collectively known as effectors, that target plant molecules and alter plant processes. It is now well accepted that in many plant–pathogen interactions, effectors are key pathogenicity determinants that modulate plant innate immunity and enable parasitic infection (Kamoun, 2007; Hogenhout *et al*., 2009). Currently, the study of the genome organization, evolution, trafficking and function of filamentous pathogen effectors is a very active area of research as evidenced by a high level of exposure at international conferences (Walton *et al*., 2009).

The concept that filamentous pathogens secrete proteins to perturb the hosts they colonize is not particularly novel, but research has accelerated because it has become possible to generate catalogues of the complete set of secreted proteins (secretome) for a given pathogen species (Dean *et al*., 2005; Kamper *et al*., 2006; Haas *et al*., 2009). This was driven by the coming of age of genome sequencing coupled with robust computational predictions of secretion signals and other sequence motifs diagnostic of effectors. Indeed, one feature of some families of filamentous pathogen effectors is the occurrence of linear motifs or short sequence patterns that are associated with a particular function, such as translocation inside host cells or targeting to host cell nuclei. In Table 1, we list and describe all the linear motifs reported to date for filamentous pathogen effectors. The most prominent example is the RXLR sequence motif that defines a host translocation domain in the RXLR family of oomycete effectors (Morgan and Kamoun, 2007; Whisson *et al*., 2007; Birch *et al*., 2008). The occurrence of the conserved RXLR motif has enabled the computational development of genome-wide catalogues of candidate RXLR effectors from the genome sequence of several oomycete pathogens (Tyler *et al*., 2006; Win *et al*., 2007; Jiang *et al*., 2008; Haas *et al*., 2009). Predicted RXLR effector genes were also used in high-throughput screens (effectoromics) to rapidly and efficiently assign them novel functional activities (Vleeshouwers *et al*., 2008; Oh *et al*., 2009). The RXLR motif has not been described in fungal effectors. Other motifs have been reported in fungal effectors but whether they function in host translocation has not been evaluated experimentally so far (Table 1).

Received 8 February, 2010; revised 17 March, 2010; accepted 22 March, 2010. *For correspondence. E-mail sophien.kamoun@tsl.ac.uk; Tel. (+44) 1603 450400; Fax (+44) 1603 450011; web: http://www.KamounLab.net.

Table 1. Sequence motifs reported in filamentous pathogen effectors.

Motif	Proteins	Pathogen(s)	Evidence/function	Reference(s)
RXLR (sometimes occurs as RXLR-dEER)	RXLR effectors: ATR1, ATR13, AVR3a, AVR1b etc . . .	*Phytophthora* spp. and downy mildews (*Hyaloperonospora arabidopsidis*)	Experimental/host cell translocation	Whisson *et al.* (2007), Dou *et al.* (2008), Grouffaud *et al.* (2008)
W-Box, L-Box, Y-Box	Superfamily of RXLR effectors: IPIO1, AVR4 etc.	*Phytophthora* spp.	Experimental/required for avirulence activity	Jiang *et al.* (2008), van Poppel *et al.* (2008), Champouret *et al.* (2009)
LXLFLAK	Crinkler (CRN) effectors: CRN1, CRN2, CRN8 etc.	*Phytophthora* spp.	None reported	Win *et al.* (2007), Haas *et al.* (2009)
HVLVXXP	Crinkler (CRN) effectors: CRN1, CRN2, CRN8 etc.	*Phytophthora* spp.	None reported	Haas *et al.* (2009)
[F/L]XLYLALK	CRN-like proteins: AeCRN5, AeCRN13	*Aphanomyces euteiches*	None reported	Gaulin *et al.* (2008)
LXLYLAXR	CRN-like proteins: PuCRN3, PuCRN10	*Pythium ultimum*	None reported	Cheung *et al.* (2008)
KECXD	PcF-toxin family (Pfam PF09461)	*Phytophthora* spp.	None reported	Nicastro *et al.* (2009)
RQHHKRx_9HRRHK[a]	Uf-Rtp1p	Bean rust (*Uromyces viciae-fabae*)	Experimental/Nuclear localization	Kemen *et al.* (2005)
G[I/F/Y]A/L/S/T]R	AvrP4, AvrP123, AvrL567, AvrM	Flax rust (*Melampsora lini*)	None/no effect on translocation or avirulence activity	Catanzariti *et al.* (2006)
[LI]xAR	AVR-Piaa (PEX33), AVR-Piz-t	Rice blast (*Magnaporthe oryzae*)	None reported	Yoshida *et al.* (2009), Li *et al.* (2009)
[RK]CxxCx_{12}H	AVR-Piaa (PEX33)	Rice blast (*Magnaporthe oryzae*)	None reported	Yoshida *et al.* (2009)
[R/K]VY[L/I]R	AVRk1 family	Grass powdery mildew (*Blumeria graminis* f.sp. *hordei*)	None reported	Ridout *et al.* (2006)

a. Putative sequence 'motif' that has only been reported in single proteins so far.

This review reports on some of the most interesting aspects of a selected set of filamentous pathogen effectors and highlights recent publications and findings. The review is meant to follow-up and complement a similarly themed article published about three years ago (Kamoun, 2007).

ATR1 and ATR13: rapidly evolving oomycete effectors promote disease, also of bacteria

Two effectors with avirulence activity are known from the downy mildew *Hyaloperonospora arabidopsidis*, an oomycete pathogen of the model plant species *Arabidopsis thaliana*. These are the secreted RXLR effectors ATR1 and ATR13 with 310 and 150 amino acids in length, which trigger RPP1-Nd/WsB and RPP13-Nd mediated resistance respectively (Allen *et al.*, 2004; Rehmany *et al.*, 2005). Co-adaptation in this interaction has shaped diverse, rapidly evolving avirulence and resistance gene alleles (Allen *et al.*, 2008; Hall *et al.*, 2009). Apart from the signal peptide and the RXLR motif characteristic for most cytoplasmic oomycete effectors, ATR13 contains a conserved heptad leucine/isoleucine repeat that is required for recognition by RPP13 (Allen *et al.*, 2008). The fact that mutations in the heptad repeat have not evolved implies that the repeat might be required for exerting the virulence activity (Allen *et al.*, 2008). In addition, avirulence activity depends on variable amino acids in the C-terminus of ATR13 (Allen *et al.*, 2008). While several alleles of ATR13 evade recognition by RPP13 alleles, ATR13-Wela3 is recognized by an unlinked resistance gene (Allen *et al.*, 2008). A metapopulation study (Hall *et al.*, 2009) revealed that only one subclade of RPP13 alleles was responsible for the known recognition activity, while the largest part of the RPP13 family is likely to recognize different effectors. Notably, at least one gene of the RPP13 family unlinked to RPP13 recognizes some alleles of ATR13 in a hitherto unknown manner, highlighting the complex interactions between avirulence and resistance gene networks (Hall *et al.*, 2009). The high diversification of ATR1 and ATR13 in *H. arabidopsidis* and the diversity of their cognate resistance genes in *A. thaliana* imply that these effectors might significantly contribute to pathogen fitness. A role of ATR1 and ATR13 in suppression of basal defence pathways was established from heterologous expression and delivery to host cells using the bacterial phytopathogen *Pseudomonas syringae* DC3000 (Sohn *et al.*, 2007). Both ATR1 and ATR13 increased virulence and decreased callose deposition in *A. thaliana* when delivered by *P. syringae* DC3000 (Sohn *et al.*, 2007).

AVR3a: distinct amino acids condition avirulence and virulence

The best-characterized *Phytophthora infestans* effector is the cytoplasmic RXLR protein AVR3a. One interesting

feature of AVR3a is its dual activity in pathogenesis. AVR3a elicits the hypersensitive response (HR) in plants expressing the R3a gene (Armstrong *et al*., 2005). But in plants that lack R3a, AVR3a suppresses cell death induced by *P. infestans* INF1 elicitin (Bos *et al*., 2006). These distinct activities can be uncoupled at the structural level. AVR3a with a deletion or mutation in its C-terminal residue, Tyrosine 147, retains the ability to activate R3a-mediated hypersensitivity but fails to suppress INF1-induced cell death (Bos *et al*., 2006; 2009). In addition, the two major alleles of *Avr3a* encode proteins that differ in two amino acids in their effector domain but have markedly different activities. AVR3a$^{K80/I103}$ but not AVR3a$^{E80/M103}$ activates R3a and is a stronger suppressor of INF1 cell death (Bos *et al*., 2006).

To gain further insights into the molecular basis for AVR3a activities, Bos *et al*. (2009) performed a saturated high-throughput mutant screen to identify important amino acids involved in the effector activites. Mutations in up to 15 different positions spread along AVR3a$^{E80/M103}$, including the polymorphic position 80 resulted in gain of recognition by R3a. The majority of these mutations affected residues predicted to be surface exposed and charged; therefore it is likely that recognition by R3a relies on protein–protein interactions rather than an enzymatic activity (Bos *et al*., 2009). Interestingly, all amino acid substitutions at position 80, except aromatic and acidic residues, can activate R3a regardless of the residue present at the polymorphic position 103 (Bos *et al*., 2009). However, AVR3a$^{E80/M103}$ mutants that gained R3a-recognition did not also gain the ability to suppress INF1 cell death. Furthermore, suppression of INF1 cell death requires specific residues at position 103 besides the negatively charged residue at position 80, suggesting more stringent structural requirements (Bos *et al*., 2009). These findings led to the conclusion that distinct amino acids condition the avirulence and virulence activities of AVR3a. The identification of plant targets of AVR3a that contribute to R3a recognition and/or INF1 cell death suppression will further clarify how this effector carries out its dual biochemical activities.

Phytophthora sojae avirulence effectors: copy number variation associated with evasion of host immunity

Qutob *et al*. (2009) recently showed that segmental duplication and intraspecific copy number variation (CNV) are prevalent among the RXLR effector genes of the soybean pathogen *Phytophthora sojae*. *Avr1a, Avr3a* and *Avr3c* of *P. sojae* represent good examples of RXLR effector genes that occur in duplicated DNA segments. All three genes are recognized by specific soybean resistance genes (*Rps1a, Rps3a, Rps3c*), and were identified using a combination of genetic mapping, transcriptional profiling and functional assays (Dong *et al*., 2009; Qutob *et al*., 2009). Remarkably, all occur in tandem arrays of duplicated DNA segments that have particular features, such as presence/absence polymorphisms and variation in transcript accumulation. In *Avr1a*, four near-identical copies of a 5.2 kb segment are present in both virulent and avirulent strains and the *Avr1a* genes are transcriptionally silenced in some virulent strains (Qutob *et al*., 2009). In other *Rps1a*-virulent strains, the two segments that carry *Avr1a* are deleted (Qutob *et al*., 2009). Five predicted open reading frames occur in each of the four 10.8 kb segment duplications that include *Avr3a*. Some *P. sojae* strains that overcome *Rps3a* soybean lines show transcriptional silencing of these four *Avr3a* copies (Qutob *et al*., 2009). In other *Rps3a*-virulent strains, three of the segments are deleted and the remaining gene is transcriptionally silenced (Qutob *et al*., 2009). In the case of *Avr3c*, evasion of immunity occurs by specific mutation in the effector domain of the AVR3c protein rather than by genome-level structural changes. Nevertheless, tandem repeats may facilitate the spread of mutations via sequence exchanges between paralogues thereby enabling an additional level of protein diversification (Dong *et al*., 2009). Given that multiple copies of effector genes have been described in several oomycete and fungal genomes (Jiang *et al*., 2006; Qutob *et al*., 2006; 2009; Ridout *et al*., 2006; Dong *et al*., 2009), it is reasonable to hypothesize that CNV contributes to pathogen fitness by increasing adaptability. However, the precise relevance of genome-level structural polymorphisms and their impact on phenotypic variation is not clearly understood yet.

Crinklers: an additional large and diverse family of oomycete effectors

Investigations of *P. infestans* secreted proteins have led to the identification of an additional family of cytoplasmic effectors besides the RXLR effectors. A high throughput functional screen revealed CRN1 and CRN2 (CRN for crinkling and necrosis), two cell death-inducing proteins that cause a leaf crinkling phenotype when expressed systemically in plants (Torto *et al*., 2003). With the availability of the *P. infestans* genome sequence, examinations of gene sparse regions revealed that besides RXLR effector genes, the CRN gene family co-populates repeat rich regions and has dramatically expanded in *P. infestans* (193 predicted genes) compared with other *Phytophthora* gene families (Haas *et al*., 2009). Expansion of this gene family has occurred through multiple gene duplication and intragenic recombination events that eventually gave rise to a large and diverse chimeric effector repertoire (Haas *et al*., 2009).

CRN effectors are modular proteins that feature a predicted N-terminal secretory leader signal, followed by a domain defined by the conserved but not invariant LXLFLAK motif (the major defining feature of CRN proteins) (Haas *et al*., 2009). Most CRNs carry after the LXLFLAK domain a DWL domain that ends with the HVLVXXP motif. Haas *et al*. (2009) proposed that recombination among CRNs, particularly after the HVLVXXP motif, generated the extraordinary diversity of C-terminal domains (up to 36 divergent amino acid sequences in *P. infestans*). Current work in our laboratory aims at testing whether the LXLFLAK domain functions in host translocation.

Analogous to several RXLR proteins, expression of some CRN C-termini (thus lacking the putative N-terminal translocation regions) inside plant cells induces cell death (Haas *et al*., 2009). However, the relevance of the CRN-induced cell death in disease remains unclear. Several CRNs carry C-termini with the D2 and DBF domains that exhibit similarity to kinases and are thought to translocate inside plant cells to alter signalling processes (Haas *et al*., 2009).

Magnaporthe oryzae avirulence effectors: discovery by genome resequencing

In the rice blast fungus *Magnaporthe oryzae*, classical effectors with avirulence activities such as Avr-Pita, PWL1, PWL2 and ACE1 were identified by map-based cloning (Stergiopoulos and de Wit, 2009). The completion of the whole-genome sequence of *M. oryzae* isolate 70-15 (Dean *et al*., 2005) should have accelerated the identification of avirulence effectors given that more than 25 *R*-genes have been identified in rice (Wang *et al*., 1994). However, lack of association between avirulence phenotypes and observed polymorphisms within the candidate effector genes mined from the genome hampered the identification of new avirulence determinants (Yoshida *et al*., 2009). Instead, Yoshida *et al*. (2009) observed high levels of presence/absence polymorphisms of PCR products using the primers designed to amplify the candidate effector genes, indicating that a significant number of avirulence determinants might be missing from isolate 70-15. Genome-resequencing using the 454 technology on *M. oryzae* field isolate Ina168, which is known to contain nine avirulence genes, enabled Yoshida *et al*. (2009) to identify a total of 1.11 Mb of DNA that is absent from the published genome sequence. Yoshida *et al*. (2009) found perfect associations of three candidate effector genes, *Pex22*, *Pex31* and *Pex33*, mined from the Ina168-specific sequences, with three avirulence phenotypes, *Avr-Pia*, *Avr-Pik/km/kp* and *Avr-Pii* respectively. Subsequent genetic transformation experiments confirmed that the effectors Pex22, Pex31 and Pex33 confer avirulence towards the rice cultivars expressing *Pia Pik/km/kp* and *Pii R*-genes respectively. *Pex22* was also independently identified to be the *Avr-Pia* by positional cloning (Miki *et al*., 2009). Pex22, Pex31 and Pex33 are small, 85, 70 and 113 amino acids in length, respectively, contain secretory signals, and are recognized in the cytoplasm of rice cells implying that they are translocated inside the plant cell during infection. Interestingly, Pex33 forms a small family containing four homologues characterized by the presence of two sequence motifs: motif-1 by [LI]x-AR[SE][DSE] similar to LxAR motif of Avr-Piz-t, which is capable of suppressing BAX-mediated cell death (Li *et al*., 2009), and motif-2 by [RK]CxxCx$_{12}$H showing similarity to C2H2 zinc finger motif involved in protein–protein interaction (Yoshida *et al*., 2009).

AvrL567: effector avirulence and virulence analysed using protein structure

The AvrL567 family of translocated effectors from the flax rust fungus *Melampsora lini* are recognized by the flax resistance (R) proteins L5, L6 and L7 *via* direct protein interaction (Dodds *et al*., 2006). This system is special because crystal structures of AvrL567-A and AvrL567-D (92% sequence identity, different patterns of R protein specificity) have been elucidated (Wang *et al*., 2007). This allowed the role of polymorphic residues involved in molecular recognition events underlying gene-for-gene resistance to be examined in the context of protein structure (Wang *et al*., 2007). Both proteins adopt the same β-sandwich fold, which has no close known structural homologues. Four key, highly polymorphic residues at positions 50, 56, 90 and 96 in AvrL567 that are critical for R protein activation, map to the surface of these effector proteins. Site-directed mutagenesis of these AvrL567 amino acids alters the pattern of R protein recognition specificity and complementary amino acid variants in the flax R proteins map to the leucine-rich repeat domain. Interestingly, the four AvrL567 residues are spread across the protein surface, suggesting that the interaction between AvrL567 and the leucine-rich repeat domain of the R proteins requires multiple contact points that have cumulative effects towards the overall interaction. This may be important in the co-evolutionary arms race to retain/develop a virulence function (of benefit to the pathogen) but evade detection by the host.

The AvrL567 structures also suggest testable hypotheses to investigate the virulence activities of these effectors. Overall, the structures share limited structural similarity with ToxA, a secreted toxin of the plant pathogenic fungus *Pyrenophora tritici-repentis* (Fig. 1). Also, the structures revealed two positively charged surface patches that could bind DNA; the proteins were subsequently shown to bind nucleic acids *in vitro*. This shows

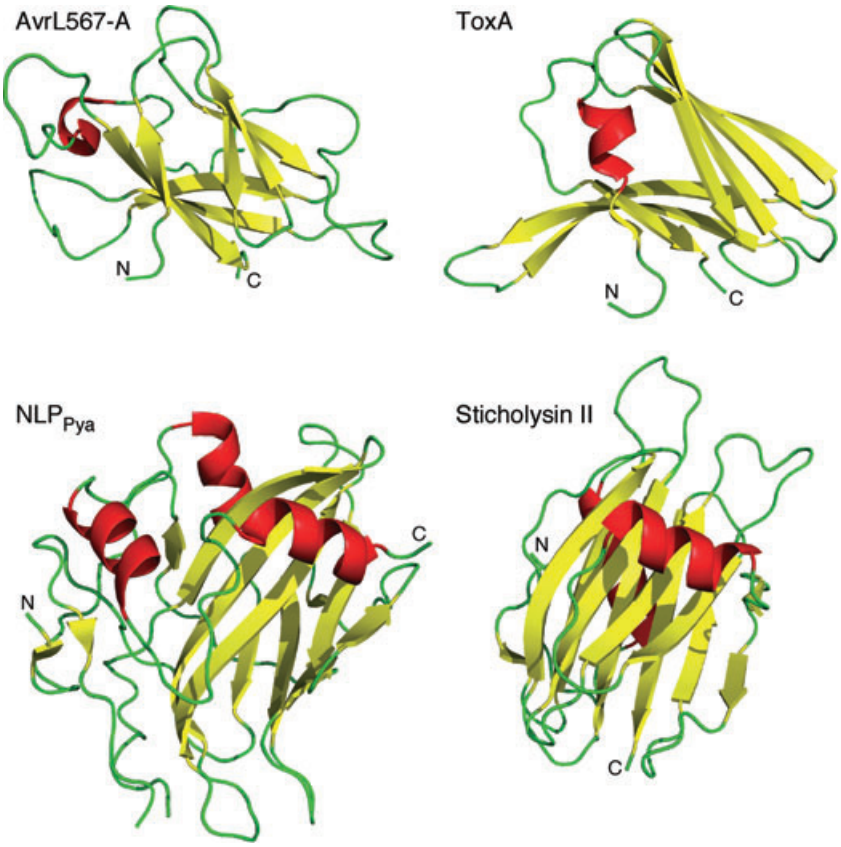

Fig. 1. Structural similarity of pathogen effectors to other proteins, identified using fold-recognition algorithms. Upper panel: Crystal structure of the flax rust effector AvrL567-A (PDB code: 2OPC) compared with *Pyrenophora tritici-repentis* ToxA (PDB code: 1ZLD). Lower panel: Crystal structure of the *Pythium aphanidermatum* Nep1-like protein NLP$_{Pya}$ (PDB code: 3GNZ) compared with the cytolytic protein of the sea anemone *Stichodactyla helianthus* Sticholysin II (PDB code: I072). Structures are coloured according to secondary structure (red = helix, yellow = beta strand, green = loop). The positions of the N- and C-termini are also labelled. Structural overlays were produced with the SSM procedure as implemented in Coot (original fold similarity identified in the appropriate publications). Figure prepared with PyMol.

how structural biology can stimulate functional studies of effectors where few clues are available from primary sequence.

AvrLm1, AvrLm6 and AvrLm4-7: effector evolution in gene-poor isochores

Map-based cloning strategies were used to clone three avirulence genes *AvrLm1*, *AvrLm6* and *AvrLm4-7* from the causal agent of stem canker on oilseed rape, *Leptosphaeria maculans* (Gout *et al*., 2006; Fudal *et al*., 2007; Parlange *et al*., 2009). They encode small putatively secreted proteins of 122 to 205 amino acids with no similarity to any protein present in public databases. AvrLm6 and AvrLm4-7 contain six and eight cysteine residues respectively. These cysteines might stabilize the AvrLm6 and AvrLm4-7 proteins in the plant apoplast. However, AvrLm1, with only one cysteine residue, is more likely to be translocated into host cells given that all apoplastic effectors described to date are cysteine-rich proteins (Kamoun, 2007). Despite their clear avirulence activities, the mechanisms of AvrLm recognition, the corresponding *Rlm* resistance genes and their site of action are still unknown. In contrast to most other fungal effector genes cloned to date, these three *L. maculans* genes share a low GC content. They all are single copy genes sitting alone in the middle of AT-rich and transposon-rich, heterochromatin-like regions of 60 kb (*AvrLm4-7*), 133 kb (*AvrLm6*) and 269 kb (*AvrLm1*). Localization of these effectors in a distinct genomic environment is reminiscent of fungal conditionally dispensable chromosomes (van der Does and Rep, 2007), sub-telomeric regions of *Plasmodium* (Pain *et al*., 2008) or gene-sparse regions in *P. infestans* (Haas *et al*., 2009). This is believed to enable faster gene evolution and accelerated adaptation of a pathogen to its host (van der Does and Rep, 2007; Pain *et al*., 2008; Haas *et al*., 2009).

Analyses of virulence in field populations of *L. maculans* provided clues about how such peculiar loci evolved to enable virulence on resistant plants. In the case of *AvrLm1*, a specific 260 kb deletion enabled gain of virulence in most of the strains virulent on *Rlm1* plants (Gout *et al*., 2007). Similarly, large deletion events spanning *AvrLm6* are responsible for gain of virulence cases on *Rlm6* plants. In addition, repeat induced point (RIP) mutations, causing dramatic sequence changes compared with avirulent alleles are also frequently observed at the *AvrLm6* locus (Fudal *et al*., 2009). Deletion of *AvrLm4-7* also seems to be the major event leading to virulence on *Rlm7* plants. However, some field isolates carry a single amino acid replacement (glycine to arginine) in AvrLm4-7 that is sufficient to escape *Rlm4*-mediated recognition

(Parlange *et al*., 2009). These studies report contrasting mechanisms of evolution towards virulence, namely gene deletion versus mutation. The degree of fitness advantage provided by the effector may explain the varying frequency of virulence mechanisms observed in field populations. In any case, genetic instability of all three *AvrLm* loci appears to be favoured by location in gene-poor isochores.

AVR1, AVR2, AVR3: virulence and avirulence determinants in the tomato xylem

Fusarium oxysporum f.sp. *lycopersici* is a vascular pathogen of tomato. Proteomics analyses of xylem sap from infected tomatoes revealed several secreted in xylem (SIX) proteins of *F. o*. f.sp. *lycopersici* (Rep *et al*., 2002; Houterman *et al*., 2007). Among these proteins, Avr1 (Six4) (Houterman *et al*., 2008), Avr2 (Six3) (Houterman *et al*., 2009) and Avr3 (Six1) (Rep *et al*., 2004) turned out to have avirulence activities. The AVR2 and AVR3 (Rep *et al*., 2005; Houterman *et al*., 2009) effectors are both required for virulence on tomato. AVR1, AVR2 and AVR3 are recognized by their cognate resistance genes from tomato, *I* (Immunity), *I-2* and *I-3* respectively. Introgression of these resistance genes from wild tomato species into commercial varieties (Huang and Lindhout, 1997) resulted in changes in the rate of occurrence of particular *F. o*. f.sp. *lycopersici* races. Introgression of the *I* gene from *Solanum pimpinellifolium* into tomato cultivars in the 1940s resulted in an increased frequency of strains lacking AVR1 and the emergence of *F. o*. f. sp. *lycopersici* race 2 (Katan and Ausher, 1974). AVR1 is not required for full virulence on tomato, but was found to interfere with AVR2 and AVR3 recognition by I-2 and I-3 (Houterman *et al*., 2008). Therefore, I-2 and I-3 confer resistance to race two strains that emerged following the introduction of tomato cultivars with the *I* gene. After the introduction of the *I-2* gene (also from *S. pimpinellifolium*) in the 1960s, another virulent race, race 3, emerged with single amino acid mutation in AVR2 indicating that virulence and avirulence can be uncoupled as discussed above for *P. infestans* AVR3a (Volin and Jones, 1982; Houterman *et al*., 2009). Finally, the *I-3* gene from *Solanum pennellii* has also been introgressed and appears to confer resistance to a broad spectrum of isolates so far (McGrath and Maltby, 1989). This relatively simple system of specific disease resistance illustrates how understanding the function and population dynamics of avirulence effectors can enhance the deployment of resistant cultivars in agriculture (Takken and Rep, 2010). For example, since AVR1 suppresses the resistance mediated by I-3, the deployment of I and I-3 in combination should prove more effective than I-3 alone. Also, because race 3 (AVR2 point mutants) is rare and has only been identified in North America and Australia (Houterman *et al*., 2009), the deployment of I-2 in other parts of the world should be relatively more effective.

Enzyme inhibitors: effectors from unrelated species inhibit the same host plant protease

Filamentous plant pathogens have evolved effector proteins with inhibitory activities for protection against several host hydrolytic enzymes (Kamoun, 2006). These effectors and host-plant targets have been recently reviewed by Misas-Villamil and van der Hoorn (2008). GIP1 (glucanase inhibitor protein-1) from *P. sojae* inhibits soybean *endo*-β-1,3-glucanase-A (Rose *et al*., 2002). GIP1 inhibition activity might impose selection pressure on the host enzyme since *endo*-β-1,3-glucanase-A is under positive selection in soybean (Bishop *et al*., 2005). The fact that GIP1 is also under positive selection in *Phytophthora* species further points to an ongoing arms race between the plant and the pathogen (Damasceno *et al*., 2008).

Cladosporium fulvum Avr2 inhibits Rcr-3 and PIP1, two closely related cysteine proteases of tomato (Krüger *et al*., 2002; Rooney *et al*., 2005; Shabab *et al*., 2008). *P. infestans* secretes an array of protease inhibitors; EPI1 and EPI10 (Extracellular protease inhibitors) target a tomato serine protease P69B (Tian *et al*., 2004; 2005), whereas EPIC1 and EPIC2B (Extracellular Cysteine Protease Inhibitors) target papain-like cysteine proteases (Tian *et al*., 2007). Both EPIC2B and Avr2 target the tomato cysteine protease PIP1 (Tian *et al*., 2007; Shabab *et al*., 2008). Also similar to Avr2, EPIC1 and EPIC2B are able to bind and inhibit Rcr3. However, unlike Avr2, they do not induce a hypersensitive response in Cf-2/Rcr3^{pim} tomato indicating that *P. infestans* evolved stealthy effectors that inhibit tomato proteases without activating immune responses (Song *et al*., 2009). The finding that unrelated pathogens evolved protease inhibitors with similar host targets, suggests that the inhibited proteases are important components of plant defence. Indeed, the rcr3-3 mutant of tomato that carries a premature stop codon in the *Rcr3* gene exhibits enhanced susceptibility to *P. infestans* (Song *et al*., 2009).

Host selective toxins: effectors from necrotrophic fungi that promote disease susceptibility in a light-dependent manner

Host selective toxins (HSTs) are chemically diverse effector molecules, which function as virulence factors produced by phytopathogenic fungi. These pathogenicity determinants are active only in the host plants that are susceptible to the pathogen from which the toxin is derived (Wolpert *et al*., 2002). Interestingly, several proteinaceous HSTs, such as PtrToxA, SnTox1, SnTox2 and

SnTox4, from the necrotrophic fungal species *Pyrenophora tritici-repentis* and *Stagonospora nodorum*, were shown to induce necrosis and promote susceptibility on toxin-sensitive host wheat plants in a light-dependent manner (Liu *et al*., 2004; Friesen *et al*., 2007; Abeysekara *et al*., 2009; Manning *et al*., 2009). Corresponding dominant host genes '*Tsn1, Snn1, Snn2, Snn4*', which are required for the functioning of these effectors, were mapped on chromosomes of toxin-sensitive hosts and the products of these genes are thought to be receptors that interact with the HSTs directly or indirectly (Liu *et al*., 2004; Friesen *et al*., 2007; Abeysekara *et al*., 2009; Manning *et al*., 2009). Remarkably, an inverse gene for gene interaction has been suggested for HSTs in which the receptor molecule is required for susceptibility rather than the resistance that is observed in classical avirulence-resistance gene interactions (Wolpert *et al*., 2002). PtrToxA is the most studied among the *P. tritici-repentis* and *S. nodorum* HSTs. It has a modular structure with an N-terminal secretion signal, which is cleaved to form the mature protein, followed by an RGD domain that is required for host translocation and a C-terminal effector domain (Sarma *et al*., 2005; Manning *et al*., 2007). PtrToxA was reported to localize into the chloroplasts and interact with the chloroplast protein ToxABP1 (Manning *et al*., 2007). Once it is translocated into chloroplasts, PtrToxA promotes virulence by interfering with photosystem I and II functioning and finally inducing reactive oxygen species accumulation in a light-dependent manner (Manning *et al*., 2009).

Nep1-like proteins: cytolytic toxins from phylogenetically unrelated microorganisms

Nep1-like proteins (NLPs) are toxins from bacteria, fungi and oomycetes that elicit necrosis in dicotyledonous plants (Pemberton and Salmond, 2004; Ottmann *et al*., 2009). Despite their diverse distribution across taxa, NLPs share a common fold characterized by a heptapeptide (GHRHDWE) motif and two conserved cysteines, showing structural similarity to actinoporins, cytolytic toxins produced by marine organisms (Gijzen and Nurnberger, 2006; Ottmann *et al*., 2009) (Fig. 1). The wide phylogenetic distribution of the NLPs indicates that they have remained conserved throughout a long evolutionary period (Fellbrich *et al*., 2002; Qutob *et al*., 2002; Ottmann *et al*., 2009). It is puzzling how such an evolutionary ancient toxin fold has been retained in diverse microorganisms with a necrotrophic or hemibiotrophic infection style (Ottmann *et al*., 2009).

In the hemibiotrophic oomycete pathogens *P. sojae* and *P. infestans*, the expression of particular NLP genes (*NPP1* and *PiNPP1.1*) is upregulated during the late necrotrophic phase of host infection (Qutob *et al*., 2002; Kanneganti *et al*., 2006). These NLPs could contribute to host tissue death with their cytolytic activity thereby facilitating colonization during necrotrophic pathogen growth (Qutob *et al*., 2002; Kanneganti *et al*., 2006). Although it is now clear that some NLPs contribute to virulence as toxins, it is unlikely that all members of this family have cytolytic toxin activities on their hosts. Unlike PiNPP1.1, two other NLPs from *P. infestans* failed to trigger cell death in plants (Kanneganti *et al*., 2006). Also, the genomes of *Phytophthora* spp. contain large numbers of NLP genes (27 to 59 and many more pseudogenes depending on the species) that may have diverse activities (Tyler *et al*., 2006; Haas *et al*., 2009). Some of the *P. infestans* NLP genes are induced during the early biotrophic phase of the disease ruling out a toxin function (Haas *et al*., 2009). The function of these NLPs remains unknown.

Nep1-like proteins do not elicit necrosis in monocotyledonous plants possibly because of a different molecular composition of the target cell membrane (Gijzen and Nurnberger, 2006). Interestingly, some monocot pathogens carry NLP genes. One example is *Mycosphaerella graminicola*, a fungal pathogen of wheat that has only one NLP gene *MgNLP* (Motteram *et al*., 2009). *MgNLP* is upregulated during wheat infection towards the end of the symptomless phase of colonization, which corresponds to the transition from biotrophy to necrotrophy. However, although MgNLP elicits cell death in *Arabidopsis*, it has no effect on its host wheat (Motteram *et al*., 2009). Targeted deletion of *MgNLP* in *M. graminicola* does not alter disease progression, indicating that this protein is not required for virulence on wheat (Motteram *et al*., 2009).

Outlook: what are the targets of filamentous pathogen effectors?

Significant progress has been made since we last reviewed the topic (Kamoun, 2007). As we stated at the time, one of the most pressing questions is to identify the biochemical activities of the effectors and to understand how they enhance the reproductive success of the pathogen. However, as this review illustrates, we still know little about the targets of filamentous pathogen effectors, particularly those effectors that are translocated inside host cells. In the near future, we can expect a flurry of discoveries on the host targets of filamentous effectors and important insights into their biochemical activities.

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