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Groovy times: filamentous pathogen effectors revealed

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Filamentous microorganisms, such as fungi and oomycetes, secrete an arsenal of effector proteins that modulate plant innate immunity and enable parasitic infection. Deciphering the biochemical activities of effectors to understand how pathogens successfully colonize and reproduce on their host plants became a driving paradigm in the field of fungal and oomycete pathology. Recent findings illustrate a diversity of effector structures and activities, as well as validate the view that effector genes are the target of the evolutionary forces that drive the antagonistic interplay between pathogen and host.

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Introduction

Filamentous microorganisms, such as fungi and oomycetes, include highly developed plant pathogens that are intimately associated with their host plants and cause a variety of disease pathologies in natural and agricultural plant communities. Until recently, our knowledge of fungal and oomycete pathogenicity was mainly limited to the development of specialized infection structures, secretion of hydrolytic enzymes, and production of host selective toxins. New findings, however, broadened our view of pathogenicity suggesting that filamentous pathogens are sophisticated manipulators of plant cells. It is now well accepted that similar to bacterial pathogens, eukaryotic pathogens secrete an arsenal of effector proteins that modulate plant innate immunity and enable parasitic infection [1–4]. Deciphering the biochemical activities of effectors to understand how pathogens successfully colonize and reproduce on their host plants became a driving paradigm in the field of fungal and oomycete pathology. The enthusiasm over effectors is further augmented by the availability of dozens of filamentous pathogen genome sequences [5], resulting in genome-wide catalogs of effector secretomes [6–8].

Molecules, currently known as effectors, have long been described by plant pathologists under other terms, including avirulence, virulence, elicitor, and toxin. Unlike these terms, the term effector is, however, neutral and does not imply a negative or positive impact on the outcome of the disease interaction. Having a neutral term such as effector is a crucial conceptual development in the field of plant pathology because many pathogen molecules have dual and conflicting functions depending on the genotype of the host and a variety of other variables. For instance, it is well established that an effector with an avirulence activity on a given host plant variety may have a virulence function on a different plant genotype [9]. An effector that triggers a necrotic response could function as an elicitor that limits infection in a biotrophic pathogen while it may facilitate infection by a necrotroph and thus function as a toxin. In hemibiotrophs like *Phytophthora*, an effector may have different effects on the interaction depending on whether it is expressed during the early biotrophic phase or the late necrotrophic stage of the disease [10–12]. This dual function of effectors is also illustrated by the underlying assumption of much current research on plant–pathogen interactions that effectors known only by their elicitor or avirulence activities have an unknown virulence activity.

The concept of 'effectors', formulated with different terminologies, has long been fascinating to biologists who pondered the theoretical concepts of host–pathogen interactions. For instance, Richard Dawkins provided what is arguably the most pertinent definition of effectors when he postulated in his classic 1982 book 'The extended phenotype' that 'it is logically sensible to regard parasite genes as having phenotypic expression in host bodies and behavior' [13]. Indeed, although effector genes reside in pathogen genomes, their products actually function at the interface with the host plant or even inside plant cells, forming one of the best examples of genes with extended phenotypes (i.e. 'whose effects reach beyond the cells where they reside') [13]. It is remarkable that Dawkins devoted an entire chapter to 'host phenotypes of parasite genes' in his book although he had access to only very few examples of parasite genes with host-extended phenotypes, most notably *Agrobacterium tumefaciens* T-DNA [13]. Much has happened since. In this review, I illustrate the remarkable progress made in studying plant pathogen genes with long reaching phenotypes with an update on exciting developments in the field of fungal and oomycete effectors. Recent findings illustrate a diversity of effector structures and activities, as well as validate the view that effector genes should be the direct targets of the evolutionary forces that drive the

antagonistic interplay between pathogen and host [13]. A description of cloned filamentous pathogen effectors is shown in Table 1. As in an earlier review, I distinguish between apoplastic effectors that are secreted into the plant extracellular space and cytoplasmic effectors that are translocated inside the plant cell [3]. Also, rather than attempting a comprehensive review, I limit the discussion to the most remarkable aspects of selected effectors (Box 1).

AvrL567, AvrM, AvrP123, and AvrP4: haustoria at work

Similar to many other filamentous plant pathogens, the obligate biotroph rust fungi produce haustoria, the specialized infection structures that develop within plant cells but remain encased in a modified plant cell membrane known as the extrahaustorial membrane [14,15]. Haustoria are thought to function in nutrient acquisition and are known to cause cellular and biochemical changes in the invaded host cell [14,15]. The haustoria of the flax rust fungus *Melampsora lini* also appear to mediate the delivery of effector proteins inside host cells [16,17•]. Four flax rust effectors, AvrL567, AvrM, AvrP123, and AvrP4, were identified among 21 haustorially expressed secreted proteins (HESPs) as having a hypersensitive elicitor activity toward their cognate flax resistance genes [17•]. This suggests that haustoria are highly enriched in secreted effector proteins and most probably play a role in mediating effector translocation into host cells.

How the flax rust effectors enhance virulence is unknown. AvrP123 is a 117 amino acid protein with similarity to Kazal serine protease inhibitors that might, therefore, target host proteases [17•]. AvrL567 is a highly

Table 1

A selection of filamentous plant pathogen effectors

Pathogen species	Effector	Localization in plant tissue[a]	Signal peptide length[b]	Positive selection	Virulence activities	Reference(s)
Blumeria graminis f. sp. *hordei*	AVRa10	Cytoplasmic	NA		Enhances infection in susceptible barley plants[c]	[29••]
	AVRk1	Cytoplasmic	NA		Enhances infection in susceptible barley plants[c]	[29••]
Cladosporium fulvum	Avr2	Apoplastic	20		Cysteine protease inhibitor; inhibits tomato Rcr3[c]	[43]
	Avr4	Apoplastic	18		Contains CBM14 chitin binding domain; protects fungal cell walls from hydrolysis by plant chitinases[c]	[44]
	Avr9	Apoplastic	23		Structural similarity to cystine knot carboxypeptidase inhibitor[d]	[57]
Fusarium oxysporum f. sp. *lycopersici*	SIX1	Apoplastic (xylem)	21			[49]
Hyaloperonospora parasitica	ATR1	Cytoplasmic	15	Yes		[30]
	ATR13	Cytoplasmic	18	Yes		[52]
Leptosphaeria maculans	AvrLm1	Probably cytoplasmic	22			[24]
Magnaporthe oryzae	Avr-Pita	Cytoplasmic	16		Metalloprotease[d]	[25]
Melampsora lini	AvrL567	Cytoplasmic	23	Yes		[16,18••]
	AvrM	Cytoplasmic	28	Yes		[17•]
	AvrP123	Cytoplasmic	23		Kazal-like protease inhibitor[d]	[17•]
	AvrP4	Cytoplasmic	28	Yes		[17•]
Phytophthora infestans	Avr3a	Cytoplasmic	21	Yes	Cell death suppressor[c]	[33••,50]
	CRN1	Cytoplasmic	17		Elicits cell death in host plants[c]	[31]
	CRN2	Cytoplasmic	22		Elicits cell death in host plants[c]	[31]
	CRN8	Cytoplasmic	17		Similarity to RD kinase[d]; elicits cell death in host plants[c]	[40], C Cakir *et al*., unpublished
	EPI1	Apoplastic	16		Kazal-like serine protease inhibitor; inhibits tomato P69B[c]	[45]
	EPI10	Apoplastic	21		Kazal-like serine protease inhibitor; inhibits tomato P69B[c]	[46]
	EPIC1	Apoplastic	21	Yes	Cystatin-like cysteine protease inhibitor[d]	[47]
	EPIC2B	Apoplastic	21	Yes	Cystatin-like cysteine protease inhibitor; inhibits tomato PIP1[c]	[47]
Phytophthora sojae	Avr1b-1	Cytoplasmic	21	Yes		[58]
Rhynchosporium secalis	NIP1	Apoplastic	20	Yes	Toxin; elicits necrosis in host plants[c]	[54]
Uromyces fabae	*Uf*-RTP1	Cytoplasmic	19		Localizes to host nucleus[c]	[19••]

[a] Cytoplasmic vs. apoplastic effectors based on the classification described in the text and in Reference [w3].
[b] Length in amino acids, based on SignalP v2.0-NN (http://www.cbs.dtu.dk/services/SignalP-2.0); NA, not applicable.
[c] Evidence is based on wet lab experimental data.
[d] Evidence is based on computational analyses.

Box 1 Freaky avirulence genes

Not all filamentous pathogen avirulence proteins are secreted effector proteins. There are two glaring exceptions. *Magnaporthe oryzae* Avirulence Conferring Enzyme1 (ACE1) is a 4035 amino acid protein that mediates avirulence on rice cultivars carrying the *Pi33* resistance gene [56]. Ace1 is a hybrid enzyme formed by a fusion between a polyketide synthase and a nonribosomal peptide synthetase, enzymes that mediate the biosynthesis of secondary metabolites in fungi [56]. Mutagenesis experiments indicated that Ace1 biosynthetic activity is required for avirulence suggesting that this enzyme probably mediates the production of an effector metabolite that triggers hypersensitivity after translocating to rice cells [56]. Another unusual case is the *Phytophthora infestans Avr3b-Avr10-Avr11* locus that controls avirulence to three potato resistance genes *R3b*, *R10*, and *R11* [26•]. The candidate avirulence gene in this locus is *pi3.4*that encodes a 1956 amino acid protein with the overall features of a transcriptional regulator, such as a WD40 domain, a nuclear localization signal, an acidic domain, and a leucine zipper [26•]. In avirulent strains, the *Avr3b-Avr10-Avr11* locus displays copy number variation resulting in an amplification of up to 25 truncated copies of *pi3.4* [26•]. Whether and how the *pi3.4* gene contributes to avirulence remains unclear in the absence of functional analyses and precise delimitation of the locus. The most plausible model, however, is that *pi3.4* confers avirulence indirectly, by regulating the expression of effector genes at other loci [26•].

polymorphic effector that binds flax L5, L6, and L7 resistance proteins in the plant cytoplasm to activate hypersensitivity and defense [16]. Diversifying selection has acted on the *AvrL567* gene, and the positively selected sites were shown to alter binding to plant resistance protein receptors providing evidence that natural selection has acted on modifying binding affinity between pathogen and plant proteins [18••].

Uf-RTP1: you're in my heart

Uf-RTP1 is a 24 kDa haustorial protein secreted by the rust fungus *Uromyces fabae* [19••]. This protein is exceptional in possessing, in addition to a classical signal peptide, a bipartite nuclear localization signal (NLS) RQHHKR[X_9]HRRHK. Expression of a green fluorescent protein (GFP) and *Uf*-RTP1 fusion protein in tobacco protoplasts demonstrated that the NLS mediates protein accumulation in plant cell nuclei [19••]. Most interestingly, *Uf*-RTP1 was detected inside infected plant cells, including host nuclei, by immunofluorescence and electron microscopy providing direct evidence that this protein translocates into host cells during colonization of bean plants by the rust fungus [19••]. This suggests that similar to some bacterial effectors like *Xanthomonas* AvrBs3 [20], filamentous pathogen effectors also target the host nucleus where they possibly alter host gene expression. Besides *Uf*-RTP1, *P. infestans* candidate effectors Nuk6, Nuk7, Nuk10, and Nuk12 were recently shown to carry a combination of signal peptide and NLS and to accumulate in plant nuclei in transient expression assays [3,21]. Nuclear localization of Nuk6, Nuk7, and Nuk10 was dependent on the plant protein importin-α suggesting that these effectors exploit host machinery to localize in the nucleus [21]. These findings are particularly interesting in view of the emerging evidence that nucleo-cytoplasmic trafficking is important for plant disease resistance response and resistance protein activity [22,23].

AvrLm1: out of nowhere

AvrLm1, a 205 amino acid protein secreted by the fungal pathogen *Leptosphaeria maculans*, mediates avirulence on *Brassica napus* plants carrying the resistance gene *Rlm1* [24]. A laborious 10-year positional cloning project was recently capped by the identification of *AvrLm1* in a gene-poor heterochromatin-like region of the *L. maculans* genome [24]. This 260 kb region is essentially composed of nested long tandem repeat (LTR) retrotransposons and is surrounded by isochores, high GC gene-rich islands. *AvrLm1* was the only predicted gene in the 260 kb region [24]. The occurrence of *AvrLm1* in such a distinct genome environment is reminiscent of the location of *Magnaporthe oryzae* avirulence gene *Avr-Pita* in a highly unstable telomeric region [25]. Also, in the oomycete *P. infestans*, the *Avr3b-Avr10-Avr11* locus displays copy number variation resulting in the amplification of multiple truncated copies of a transcription factor-like gene in avirulent strains [26•]. Localization of effector genes in regions with high genome plasticity is likely to increase genetic and epigenetic variations perhaps resulting in accelerated evolution [25]. In *Plasmodium*, genes for host-translocated effectors are often found near chromosomal ends, possibly because this position favors rapid adaptation to the host [27,28].

The origin of *AvrLm1* remains mysterious. This gene has a lower GC content compared with linked genes and is absent in virulent races of *L. maculans* as well as isolates of related species [24]. Although the function of *AvrLm1* was confirmed by transformation into a virulent strain, Rlm1-specific elicitor activity has not been demonstrated yet [24]. Subsequently, it remains unclear whether AvrLm1 functions in the apoplast or inside plant cells. This protein, however, carries a single cysteine residue and as a result differs from apoplastic effectors that are typically cysteine-rich (Table 1).

AVRa10 and AVRk1: no signal required

The *AVRa10* and *AVRk1* genes of the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* were first identified as candidate genes by positional cloning, and then shown to trigger hypersensitive cell death when expressed in the cytoplasm of barley cells carrying the *Mla10* and *Mlk1* resistance genes, respectively [29••]. The predicted AVRa10 and AVR_{k1} proteins are remarkable in being the only proteins among filamentous pathogen effectors to lack a typical secretion signal peptide [29••] (Table 1). How these proteins are secreted by the fungus is unclear but most probably involves alternative secretory pathways. Transient expression experiments of AVRa10

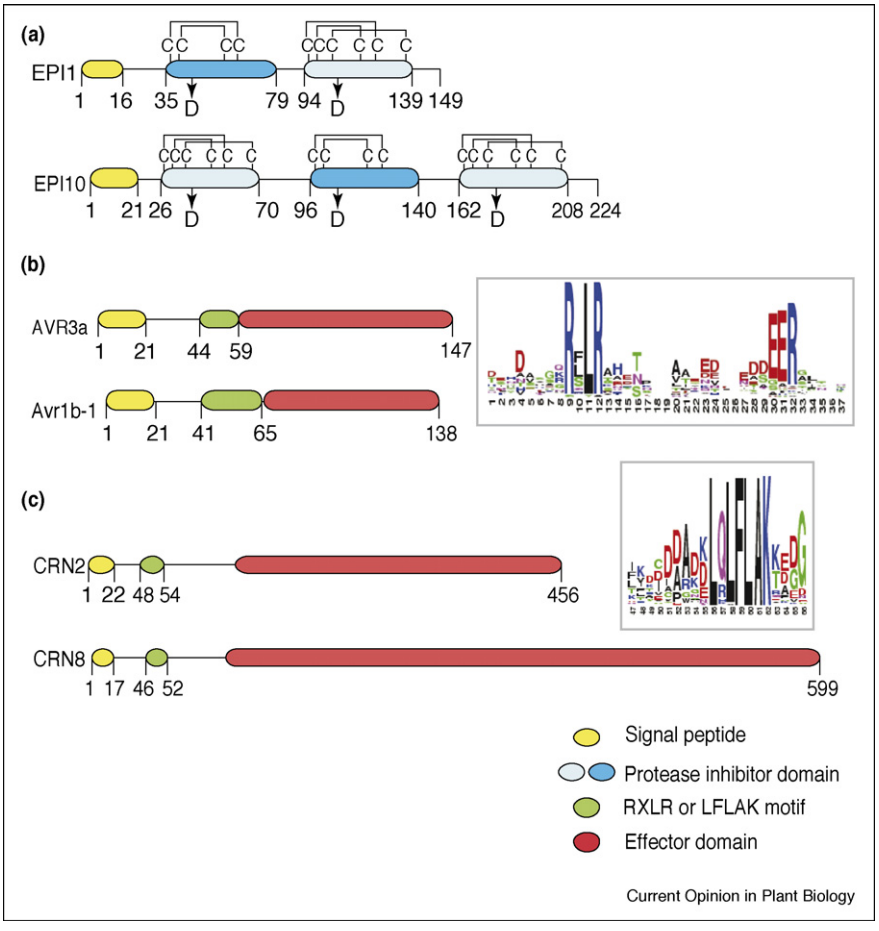
and AVR_{k1} in susceptible barley cells indicated that these effectors increase the number of successful infection sites suggesting a virulence function of an unknown nature [29••].

RXLR effectors: more than I can bear

The oomycete effectors ATR1, ATR13, AVR3a, and AVR1b are defined by an N-terminal motif RXLR (arginine, any amino-acid, leucine, arginine) and are thought to be delivered inside plant cells where they alter host defenses [1,3,30]. Genome-wide catalogs of RXLR effectors, generated using computational approaches, unraveled a remarkably complex and divergent set of hundreds of candidate genes. Tyler *et al.* [8] reported 350 RXLR effectors each in the genomes of *P. ramorum* and *P. sojae* using iterated similarity searches. Analyses in my own laboratory, using combinations of motif and hidden Markov model searches, uncovered at least 50 candidates in the downy mildew *H. parasitica* and more than 200 each in *P. capsici*, *P. infestans*, *P. ramorum*, and *P. sojae* (S Kamoun *et al.* abstract 188, XXIV Fungal Genetics Conference, Pacific Grove CA, March 2007). These large numbers of effectors suggest that oomycetes extensively modulate host processes during infection. They also raise technical challenges for studying RXLR effectors and call for implementing high-throughput functional analyses [31,32].

RXLR effectors appear to be modular proteins with two main functional domains [3,33]. While the N-terminal domain encompassing the signal peptide and conserved RXLR region functions in secretion and targeting, the remaining C-terminal domain carries the effector activity and operates inside plant cells (Figure 1). Several

Figure 1



The modular structure of oomycete effectors. (**a**) Schematic drawings of the apoplastic effectors EPI1 and EPI10 of *Phytophthora infestans*. The two-disulfide bridge Kazal domains of EPI1 and EPI10 that target the tomato protease P69B are shown in dark blue. P1 residues that are crucial for the inhibitor specificity are marked by an arrow. (**b**) Schematic drawings of the cytoplasmic RXLR effectors AVR3a of *P. infestans* and Avr1b-1 of *Phytophthora sojae*. The logo represents the RXLR-dEER motif based on an alignment of 46 oomycete proteins. (**c**) Schematic drawings of the cytoplasmic CRN effectors CRN2 and CRN8 of *P. infestans*. The logo represents the LFLAK motif based on an alignment of 105 oomycete proteins. In all panels, the numbers under the sequences indicate amino-acid positions. For clarity the CRNs were drawn to half the scale of the other proteins.

independent lines of evidence suggest that the RXLR motif defines a domain that functions in the delivery of the effector proteins into the host cell. For instance, the RXLR motif is similar in sequence and position to the plasmodial host translocation (HT)/Pexel motif that functions in the delivery of parasite proteins into the red blood cells of mammalian hosts [27,34]. Also, the RXLR domains of *P. infestans* AVR3a and another RXLR protein PH001D5 are able to mediate the export of the green fluorescent protein (GFP) from the *Plasmodium falciparum* parasite to red blood cells [35••] suggesting that plant and animal eukaryotic pathogens share similar secretory signals for effector delivery into host cells [36].

The *P. infestans* RXLR effector Avr3a is able to suppress hypersensitive cell death induced by another *P. infestans* protein, INF1 elicitin, pointing to a possible virulence function [33••]. The occurrence of cell death suppressing effectors has been hypothesized for biotrophic fungal and oomycete pathogens [37] based on cytological observations of susceptible interactions and the prevalence of cell death suppressors among bacterial type III secretion system (TTSS) effectors [38,39]. To date Avr3a, however, is the only cell death suppressor described in filamentous pathogens. The extent to which other effectors can suppress cell death is a subject of intense investigation.

CRNs: new kids in town

In addition to the RXLR effectors, which were the main focus of attention in the past two years, oomycetes also secrete CRN ('Crinkler') proteins, a second class of cytoplasmic effectors that were shown to alter host responses and may play equally important functions [3,31]. CRN1 and CRN2 were identified following an *in planta* functional expression screen of candidate-secreted proteins of *P. infestans* (Pex) on the basis of a vector derived from *Potato virus X* [31]. Expression of both genes in *Nicotiana* spp. and in the host plant tomato results in a leaf-crinkling and cell-death phenotype accompanied by an induction of defense-related genes. Database searches revealed that the CRNs form a complex family of relatively large proteins (about 400–850 amino acids) in *Phytophthora* [40]. One of the CRN-like proteins, CRN8, is predicted to be a secreted protein with a RD kinase domain that appears to target host factors and perturb host defense responses [40] (C Cakir and S Kamoun, unpublished). Interestingly, a secreted kinase was recently shown to be a major virulence factor in the apicomplexan parasite *Toxoplasma gondii* (a relative of *Plasmodium*) [41,42] further strengthening the view that oomycetes and apicomplexans share similar infection mechanisms [36].

Deletion analyses suggest that similar to the RXLR effectors, the CRNs are also modular proteins with the effector activities encoded by the C-terminal region [3] (C Cakir *et al.*, unpublished results) (Figure 1). Multiple alignments of oomycete CRN proteins, including sequences predicted from 16 *P. infestans* full-length cDNAs [40], revealed a highly conserved motif, LXLFLAK, in the region downstream of the signal peptide (Figure 1). Whether this motif, like the RXLR domain, functions in host translocation remains to be determined.

Enzyme inhibitors: counter punch

Filamentous plant pathogens have evolved effector proteins with inhibitory activities for protection against host hydrolytic enzymes, such as proteases, glucanases, and chitinases [3,43,44]. Among these *P. infestans* EPI1 and EPI10 are multidomain-secreted serine protease inhibitors of the Kazal family that bind and inhibit the pathogenesis-related (PR) protein P69B, a subtilisin-like serine protease of tomato that functions in defense [45,46]. *P. infestans* also secretes the cystatin-like cysteine protease inhibitors EPIC1 and EPIC2B that target PIP1 and other apoplastic cysteine proteases of tomato [47]. PIP1 is closely related to tomato Rcr3, another apoplastic cysteine protease that is required for Cf-2-mediated resistance to the fungus *Cladosporium fulvm* and is inhibited by the fungal Avr2 protein [43,48].

Six1: vein melter

Six1 is a 32 kDa cysteine-rich protein secreted by the fungus *Fusarium oxysporum* f. sp. *lycopersici*, a vascular pathogen of tomato [49]. In infected tomato plants, Six1 accumulates in the xylem where it is processed by either fungal or plant proteases into a 12 kDa protein [49]. Six1 mediates avirulence to tomato plants carrying the resistance gene *I-3*, as shown by complementation and knockout experiments [49]. A direct cell death elicitor activity, however, has not been reported for Six1. Presumably, Six1 interacts with a receptor at the surface of the xylem parenchyma cells to trigger hypersensitive cell death, but cloning of the *I-3* gene is needed to help clarify this issue [49].

Effector evolution: your latest trick

Effector genes are expected to be the direct target of the evolutionary forces that drive 'arms race' coevolution between pathogen and host because their phenotypes extend to plant cells and tissues [13]. Effector alleles that increase the reproductive success of the pathogen will be immediately favored by natural selection and positively selected. Indeed, many effector genes have evolved at accelerated rates compared with the remainder of the pathogen genome. For instance, the RXLR effector genes *Avr3a* of *P. infestans* and *ATR1* of *H. parasitica* occur in syntenic chromosomal regions but are drastically divergent in primary sequence [50]. Similar patterns of rapid evolution at RXLR effector loci were also reported for *P. sojae* and *P. ramorum* [8,51]. In addition, several filamentous pathogen effectors show robust evidence for positive selection (Table 1) [17•,18••,30,52–54]. In modular proteins, such as oomycete RXLR effectors, the

different domains appear to be under different selective pressures. For example, positive selection has for the most part targeted the C-terminal effector domain rather than the signal peptide and the RXLR regions [30,52] (S Kamoun *et al*. abstract 188, XXIV Fungal Genetics Conference, Pacific Grove CA, March 2007). This is reminiscent of bacterial Type III secretion system effectors, in which different domains are under different selection pressures [55].

Outlook—effector virulence functions: ray of light

One of the most pressing questions in the study of effectors is to identify their biochemical activities to understand how they perturb plant processes and increase the reproductive success of the pathogen. As mentioned above, some effectors, such as the protease inhibitors, have obvious functions, but the virulence activities of the majority of filamentous pathogen effectors remain unknown (Table 1). In the future, understanding the perturbations caused by filamentous pathogen effectors will help to unravel mechanisms of pathogenicity as well as further illuminate mechanisms of plant defense and innate immunity.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pbi.2007.04.017.

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