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How do oomycete effectors interfere with plant life?

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Oomycete genomes have yielded a large number of predicted effector proteins that collectively interfere with plant life in order to create a favourable environment for pathogen infection. Oomycetes secrete effectors that can be active in the host's extracellular environment, for example inhibiting host defence enzymes, or inside host cells where they can interfere with plant processes, in particular suppression of defence. Two classes of effectors are known to be host-translocated: the RXLRs and Crinklers. Many effectors show defence-suppressive activity that is important for pathogen virulence. A striking example is AVR3a of *Phytophthora infestans* that targets an ubiquitin ligase, the stabilisation of which may prevent host cell death. The quest for other effector targets and mechanisms is in full swing.

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Introduction

Oomycetes are fungal-like microorganisms that belong to the kingdom Stramenopila and are evolutionary related to brown algae. Many well-known plant pathogens are oomycetes, ranging from the necrotrophic broad host range *Pythium* species, through hemibiotrophic *Phytophthora* species, to obligate biotrophic species such as white rust (*Albugo*) and narrow host-range downy mildews (e.g. *Hyaloperonospora* and *Bremia* species). These oomycete pathogens interfere with plant life for successful infection, utilising effector proteins to manipulate their hosts. The sequencing of the genomes of *Phytophthora ramorum* and *P. sojae* [1], *P. infestans* [2••], *Pythium ultimum* [3••], and *Hyaloperonospora arabidopsidis* [4••] has advanced the field enormously by enabling the identification of a large number of genes encoding secreted effector proteins in this diverse group of pathogens (Figure 1). In *Phytophthora* species many effector genes are found in gene-poor regions that have recently expanded, probably driven by repeats (transposons) [2••,5]. These regions are linked to accelerated gene evolution after host jumps [5] and may explain the evolutionary flexibility of these species. Also, more novel combinations of known protein domains are present in oomycetes than in other species with a similar single domain repertoire. These combinations are enriched in secreted infection-related proteins, suggesting that oomycetes have evolved a unique toolbox for host manipulation [6•,7].

Here we discuss (summarised in Figure 2) recent advances in understanding the roles of secreted oomycete effectors in successful infection of host plants.

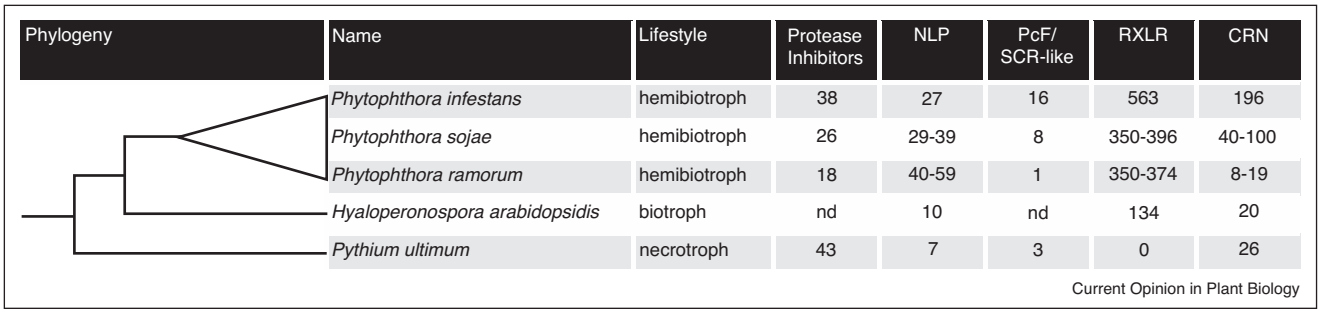
Clearing the way

In early phases of the interaction, the invading oomycete needs to deal with biochemical barriers in the plant apoplast. Both pathogen and host secrete proteins and metabolites to control the extracellular environment. Three types of apoplastic effectors from oomycetes have been shown to interfere with plant processes; inhibitors of host enzymes, RGD (Arginine–Glycine–Aspartic acid)-containing proteins, and toxins that lead to host cell death.

Enzyme inhibitors counter hydrolytic enzymes (e.g. chitinases, glucanases, and proteases) that are secreted by the host. Plant proteases contribute to pathogen defence, for example susceptibility of *Nicotiana benthamiana* to *P. infestans* is increased when the apoplastic protease *C14* genes are silenced [8•]. Furthermore, tomato cysteine protease *rcr3* mutants are more susceptible to *P. infestans* infection than wild-type tomato [9•]. Inhibiting apoplastic proteases is therefore an effective virulence strategy of pathogens. Rcr3 activity is reduced by the extracellular protease inhibitors EPIC1 and EPIC2B, which also inhibit other proteases, for example C14 [8•,9•] and PIP1 [10]. The EPICs show signs of recent evolution in *P. infestans* [10], and also its host target, potato C14, is under diversifying selection [8•], indicating these apoplastic effectors are locked in an evolutionary arms race. Whether oomycetes secrete inhibitors to arm themselves against host hydrolytic enzymes or to protect their secreted proteinacious effectors from degradation remains to be determined.

A second type of apoplastic effectors interferes with adhesion, and possibly signalling, between host cell wall and plasma membrane, for example IPI-O of *P. infestans* that seems to act at two cellular locations; inside the host

Figure 1

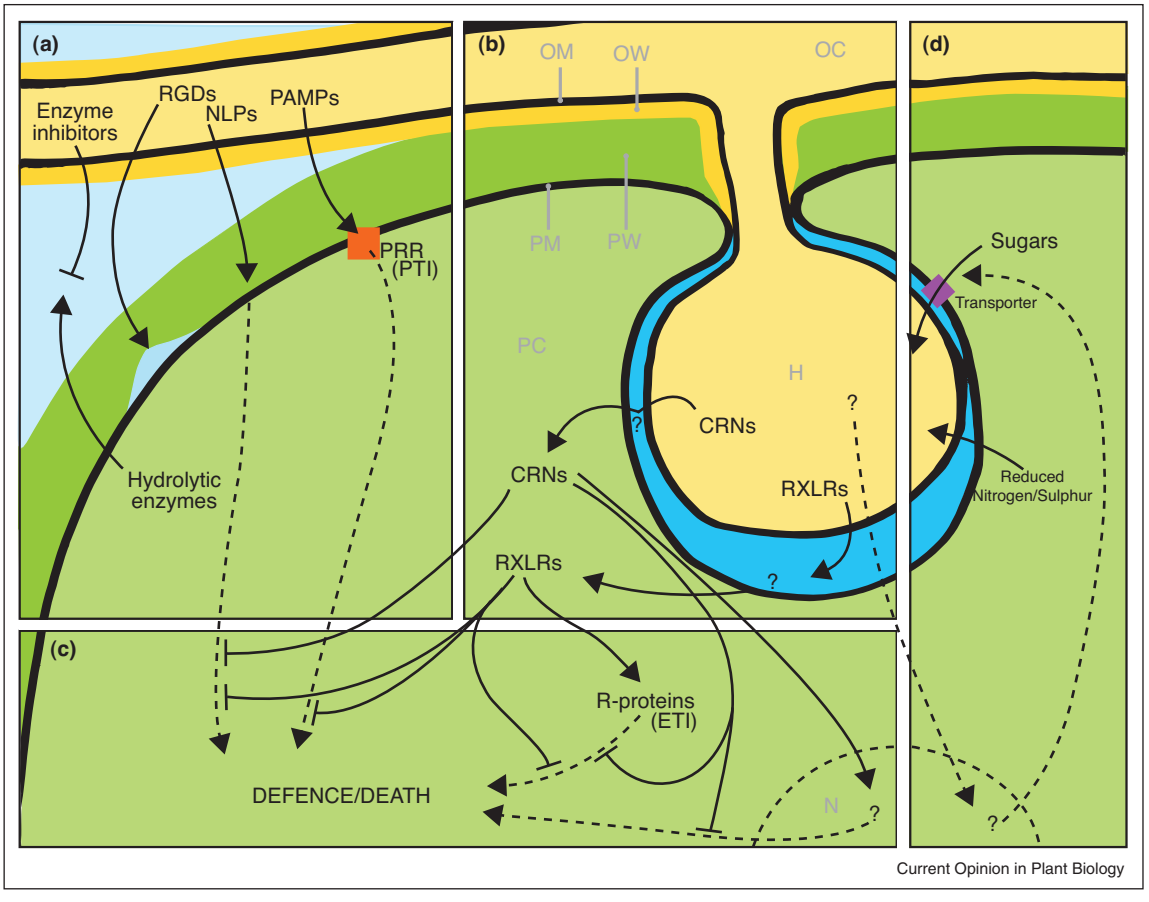


Phylogeny	Name	Lifestyle	Protease Inhibitors	NLP	PcF/ SCR-like	RXLR	CRN
	Phytophthora infestans	hemibiotroph	38	27	16	563	196
	Phytophthora sojae	hemibiotroph	26	29-39	8	350-396	40-100
	Phytophthora ramorum	hemibiotroph	18	40-59	1	350-374	8-19
	Hyaloperonospora arabidopsidis	biotroph	nd	10	nd	134	20
	Pythium ultimum	necrotroph	43	7	3	0	26

The lifestyle of oomycetes of which a full genome sequence is available and the number of members per family of selected effector types reported [1,2••,3••,4••]. The presumed evolutionary relationship between the species is indicated in the phylogenetic tree.

cell, to suppress defence, and extracellularly. The IPI-O RGD-motif disrupts the adhesion between cell wall and plasma membrane [11], possibly by binding to the Arabidopsis legume-like lectin receptor kinase LecRK-I.9 [12]. Arabidopsis *lecRK-I.9* mutants and *IPI-O* overexpression lines are more susceptible to *Phytophthora brassicae*, are altered in cell wall-integrity, and impaired in callose deposition [13]. IPI-O mediated disruption of plasma

Figure 2



Schematic representation of how oomycetes interfere with plant life. Details are described in the main text under the headings; **(a)** Clearing the way, **(b)** Breaking in, **(c)** Quelling resistance, and **(d)** Sweet rewards. Proteins produced in the oomycete cytoplasm (OC) are secreted over the oomycete membrane (OM) and cell wall (OW). The haustorium (H) has breached the plant cell wall (PW) and invaginated the host plasma membrane (PM). RXLR and Crinkler effectors (RXLRs and CRNs) are secreted from the haustorium and arrive, for example in the plant cytoplasm (PC) and nucleus (N) where they exert their activity on host cell processes.

membrane-cell wall contacts could interfere with cell-wall-associated defences, thereby promoting infection.

A third type of apoplastic effectors represents toxins that are produced by oomycetes that are necrotrophic (e.g. certain *Pythium* species) or hemibiotrophic (most *Phytophthora* species). These toxins act in an offensive way by triggering host cell death that could favour the necrotrophic phase of development. Two families of toxic proteins are encoded in the genomes of most oomycetes; the PcF/SCR proteins that are small, secreted, hydroxyproline-containing proteins, and the NEP1-like proteins (NLPs), that are related to the necrosis and ethylene inducing peptide (NEP1) from the fungus *Fusarium oxysporum*. NLPs can induce cell death in dicots by acting on the outside of the host cell membrane, as shown for NLP_{Pp} of *Phytophthora parasitica* [14]. The crystal structure of NLP_{Pya} from the necrotroph *Pythium aphanidermatum* revealed structural homology to cytolytic actinoporins [15], suggesting that NLPs insert into the host membrane to form pores. The cytolytic activity could be responsible for the necrotic response, however, cell death induction by NLPs requires host defence signalling and active host metabolism [14,16]. It was surprising to find a family of NLP genes in the obligate biotroph *H. arabidopsidis*, as this pathogen is dependent on living plant cells and does not induce host cell death. One of nine *H. arabidopsidis* NLPs (HaNLP3) belongs to the clade of necrosis-inducing NLPs, but is not capable of inducing cell death [4••]. Also *Phytophthora* species have many non-cytolytic NLPs that, like HaNLP3, could have an alternative function. One proposed alternative function is attachment to the host, as NLP_{Pya} also shows structural homology to fungal lectins that could bind surface-exposed compounds such as glucans [15].

Breaking in

The (hemi)biotrophic oomycetes engage in an intimate relation with plant cells by forming haustoria that serve a dual role; nutrient uptake from the host and delivery of effectors to the host. Haustoria penetrate the host cell wall, invaginate the host membrane, contain specific membrane proteins required for pathogenicity [17], and have been implicated as a site of effector production and secretion [18]. Effectors are secreted from the pathogen, and those that carry host-translocation signals are transported into the plant cell.

Two classes of host-translocated effectors are currently known in oomycetes. The first, the RXLR-effectors, are named after a four amino acid (Arginine, any amino acid, Leucine, Arginine; in short: RXLR) motif that was found in 2005 to be common among all then known oomycete avirulence (AVR) proteins, which are recognised inside the host cell [19]. The RXLR-effectors have an N-terminal domain consisting of a signal peptide, an RXLR-like motif, an optional amino acid motif (consisting of two glutamic acid residues and an arginine residue, often preceded by an aspartic acid residue) known as the dEER-motif, and a C-terminal effector domain. A stunning 563 RXLR-effectors are predicted for *P. infestans* [2••], whereas they are absent in *Pythium ultimum* [3••] and *Aphanomyces euteiches* [20], suggesting these effectors have recently evolved within the Peronosporales [3••].

RXLR-effectors of different species show extensive sequence divergence, though in *P. sojae* and *P. ramorum* most have been suggested to belong to a single superfamily [21]. There is little overlap in the *RXLR* gene repertoire between the different sequenced *Phytophthora* species, but also compared to *H. arabidopsidis*, suggesting recent species-specific evolution and expansion [21]. Accelerated evolution of effector genes was confirmed by analysis of genomes of 4 *P. infestans* sister species [5].

The RXLR-motif has been shown to be involved in translocation into the host [18,22]. A possible mechanism by which this occurs is by binding of the motif to phosphatidylinositol-3-phosphate (PI3P) and subsequent lipid-raft dependent uptake [23•]. However, this mechanism and its supporting experiments are strongly debated. Other mechanisms of binding/entry of effectors, for example via other negatively charged molecules, are being proposed but are, however, not yet published. Entry after binding phospholipids has been shown for effectors from different pathogens, including fungi [23•]. However, for one of the tested fungal effectors, AvrL567, lipid binding could not be reproduced by another laboratory, though background binding of phospholipids was observed [24]. Such lipid binding assays appear technically demanding and condition-dependent, warranting caution when interpreting their results. Furthermore, lipid binding properties of AvrM can be separated from the sequence required for uptake [24]. These observations on AvrL567 and AvrM do not support the idea that lipid binding by the RXLR-like amino acid motifs is required for translocation of fungal effectors. Indications that the RXLR-motif may not be essential for uptake of oomycete effectors are found in effectors of *Pseudoperonospora cubensis*, in which the EER-motif has a more important role than the RXLR-motif or RXLR-like-motif QXLR, which is prevalent in this species [25]. Furthermore ATR5 of *H. arabidopsidis* has no clear RXLR-motif, but does have an EER-motif and is translocated as it is recognised intracellularly [26]. More examples of variants of the RXLR-motif or EER-motif found in proteins that can translocate into the host cell will undoubtedly emerge in the near future, enlarging the known effector arsenal of oomycete species even further.

The second class of host intracellular effectors encompasses the Crinklers. These effectors have conserved N-termini, which contain a signal peptide, an LXLFLAK-motif followed by a conserved DWL-domain and an

HVLVXXP-motif [2••]. The LXLFLAK-motif has been shown to be required for host intracellular localisation of the Crinklers, as this N-terminal motif, but not mutated forms, enable transport of the C-terminal half of AVR3a into the plant cell, where AVR3A triggers cytoplasmic recognition in the presence of the resistance protein R3a [27••]. Like the RXLR-effectors, the modular C-terminal effector domains of the Crinklers are highly diverse. Several C-terminal domains of *P. infestans* Crinklers have been shown to target the host nucleus, and/or induce cell death when expressed within plant cells [2••,27••].

The rapid evolution and expansion of genes encoding host-translocated effectors stresses the presumed importance of these proteins in establishing a successful infection. The positive selection, which is observed for many effectors, highlights the evolutionary adaptation to overcome the immune system of the plant.

Quelling resistance

The suppression of plant defence, that is PAMP-triggered immunity (PTI) that is activated upon invasion of pathogens (see Box 1), is key to successful infection of the host. This is well documented for bacterial pathogens that use host-translocated (type III-secreted) effectors to interfere with host defence responses. It is evident that oomycete pathogens actively suppress innate immunity too. A striking example is the suppression of host cell death in the Arabidopsis lesion mimic mutant *lsd1* by the white rust pathogen *Albugo candida* [32]. Moreover, for many effectors that have been predicted from oomycete genomes a putative defence-suppressive or susceptibility-inducing activity has been found. However, the molecular mechanisms by which host-translocated effectors interfere with plant life are still largely unknown. Table 1 gives an overview of oomycete host-translocated effectors that are active in plant cells, either as suppressors or as inducers of defence.

Box 1 Plant defence in brief

A first line of activated plant defence against invading microorganisms is triggered by pathogen-associated molecular patterns (PAMPs) that are recognised by pattern recognition receptors (PRRs), inducing PAMP-triggered immunity (PTI) [28]. Oomycetes secrete several proteins that can act as PAMPs, for example elicitins, transglutaminase, and cellulose-binding proteins [29]. Oomycete cell wall derived fragments, for example β-glucan fragments, can also act as PAMPs [30]. The BAK1 PRR co-receptor is required for immunity to *P. infestans* in *Nicotiana benthamiana* [31], however, no plant genes encoding PRRs that detect oomycete PAMPs have been cloned so far. Pathogens have evolved effector proteins to suppress PTI and have thereby established effector-triggered susceptibility (ETS). Plants have evolved resistance proteins to recognise these effectors or their activity. This second line of defence, named effector-triggered immunity (ETI), confers race-specific resistance within plant species and can be very effectively phenotyped. This has been instrumental in the cloning of many oomycete effector genes (see Table 1), which are also named avirulence (AVR) genes as they confer incompatibility on host plants that carry the corresponding resistance (R) gene.

A challenging goal is to determine the function of oomycete effectors, as the availability of powerful bioassays is limited. Most currently used assays monitor the suppression of PTI. An elegant method is the use of *Pseudomonas* bacteria for type III delivery of oomycete effectors in the host. Bacteria that translocate the *H. arabidopsidis* ATR1 and ATR13 proteins were able to grow to higher densities indicating that susceptibility of the plant was enhanced [33]. ATR13 suppresses PTI as transgenic plants showed reduced PAMP-triggered callose deposition [33]. A similar activity was observed for the RXLR29 protein, that is an isolate-specific effector of *H. arabidopsidis* [34]. Another method to suppress PTI responses is by *Agrobacterium*-mediated transient expression of effector genes. Co-expression of the *P. infestans* AVR3a protein was shown to suppress PTI-associated cell death induced by the elicitin INF1 [35]. Expression of the C-terminal domain only, without signal peptide and RXLR-DEER domain, was sufficient for AVR3a effector function. An alternative way to test effectors *in planta* is by particle bombardment, for example by monitoring the suppression of cell death induced by the mouse pro-apoptotic protein BAX. Reduction of cell death was observed by co-bombardment with the *P. sojae AVR1b* gene which encodes a protein with conserved C-terminal W and Y motifs that are present in many *Phytophthora* effectors [36]. These motifs are required for suppression of cell death, suggesting that this activity is a major function of oomycete effectors. Cell death-suppression assays have been used by many groups to test candidate effectors. A screen of 32 *P. infestans* RXLR effectors for suppression of elicitin INF1-induced cell death was used to reveal effector activity of the PexRD8 and PexRD36$_{45\text{-}1}$ RXLR proteins [37]. One would expect that the suppression of defence is vital to the virulence of oomycetes, however, *in vivo* data that support this idea are missing for most effectors as knocking out genes is not an established method in oomycete research. Nevertheless, gene silencing of *Avr3A* in *P. infestans* [38•], and of the Crinklers *PsCRN115* and *PsCRN63* in *P. sojae* resulted in reduced virulence [39].

Other RXLR effectors are able to suppress cell death that is associated with effector triggered immunity (ETI, see Box 1). IPI-O4, a sequence divergent member of the *P. infestans* IPI-O family, can suppress cell death induced by variants of IPI-O1 and IPI-O2 (now renamed to AVRblb1) in potato plants carrying the resistance gene *Rpi-blb1* [40,41]. A broad ETI suppressive activity was detected for suppressor of necrosis 1 (SNE1), a *P. infestans* protein with a RXLR-like motif (RQLG) [42•]. SNE1 was able to suppress ETI triggered by the recognition of avirulence proteins from oomycetes (AVR3a), bacteria (AvrPto), fungi (Avr9) and viruses (PVX coat protein).

Table 1

Host-translocated effectors of oomycete pathogens with described *in planta* effects. Class: CRN; Crinkler effector, RXLR; RXLR-motif effector, RXLR?; RXLR-like motif effector. ETI: corresponding *in planta* R–gene. Species (Sp.): Ae; *Aphanomyces euteiches*, Ha; *H. arabidopsidis*, Pi; *P. infestans*, Ps; *P. sojae*

Effector	Sp.	Class	ETI	Effects	Target	Reference
AeCRN5	Ae	CRN		Cell death (via nucleus)		[27••]
ATR1	Ha	RXLR	AtRPP1	Contributes to virulence		[19]
ATR5	Ha	?	AtRPP5			[26]
ATR13	Ha	RXLR	AtRPP13	Suppresses callose deposition and ROS secretion		[49]
AVR1	Pi	RXLR	R1			[50]
AVR2	Pi	RXLR	R2			[50]
AVR3a	Pi	RXLR	R3a	Stabilises host E3-ligase	CMPG1	[38•,51]
AVR3b/10/11	Pi	RXLR	R3b/R10/R11			[52]
AVR4	Pi	RXLR	R4			[53]
AVRBlb2	Pi	RXLR	Rpi-Blb2	Interferes with protease secretion		[50]
IPI-O1	Pi	RXLR	Rpi-Blb1/Rpi-Sto1/ Rpi-Pta1	Disruption PM-CW integrity	LecRK-I.9	[40,12,13]
IPI-O2	Pi	RXLR	Rpi-Blb1/Rpi-Sto1/ Rpi-Pta1			[40]
IPI-O4	Pi	RXLR		Suppresses IPI-O1/IPI-O2 ETI		[41]
$PexRD36_{45-1}$	Pi	RXLR		Suppresses INF1 PTI		[37]
PexRD8	Pi	RXLR		Suppresses INF1 PTI		[37]
SNE1	Pi	RXLR?		Suppresses Avr3a ETI and NLP-induced cell death		[42•]
Various CRNs (e.g. CRN 1,2,8,16)	Pi	CRN		Cell death (via nucleus)		[2••,27••]
AVR1a	Ps	RXLR	Rps1a			[54]
AVR1b-1	Ps	RXLR	Rps1b	Suppresses BAX-induced cell death		[36,55]
AVR1k	Ps	RXLR	Rps1k			[50]
AVR3a	Ps	RXLR	Rps3a			[54]
AVR3c	Ps	RXLR	Rps3c			[56]
AVR4/6	Ps	RXLR	Rps4/Rps6			[57]
PsCRN115	Ps	CRN		Suppresses PsCRN63/NLP-induced cell death		[39]
PsCRN63	Ps	CRN		Cell death (via nucleus)		[39]

Furthermore, it also suppressed NLP-induced cell death. As *SNE1* expression is high during the biotrophic stage, and drops when *NLP* expression rises, SNE1 could be important in timing the onset of the necrotrophic phase.

For most of the effectors discussed a virulence target in the host cell has not yet been identified. An exception is the well studied *P. infestans* AVR3a protein that targets a host ubiquitin E3-ligase, CMPG1 [38•]. CMPG1 is one of three host ubiquitin E3-ligases known to be involved in plant defence [43]. AVR3a manipulates the host ubiquitin proteasome system by stabilising CMPG1 and suppresses plant immunity. More oomycete effectors are expected to act on the ubiquitination systems of the host, however, recognisable domains are absent in predicted host-translocated effectors. Effectors could have structural similarities to ubiquitination-related proteins, for example the bacterial AvrPtoB effector that was recognised as an E3 ligase based on its structure, rather than homology [44].

Sweet rewards

Interfering with plant metabolism is another anticipated activity of oomycete pathogens, especially in biotrophic interactions. In contrast to hemibiotrophs, that switch to necrotrophy in the course of infection possibly by using NLPs and other effectors that trigger cell death, obligate biotrophs do not kill their host cells, but rather keep plants cells alive while they retrieve nutrients from the host. Analysis of metabolic pathways, based on predicted oomycete proteomes has confirmed that the *Phytophthora* and *Pythium* species are autotrophic (besides their requirement of sterols) and can hence make use of a broad range of nutrients that are available in the host [1,2••,3••]. By contrast, the obligate biotroph *H. arabidopsidis* lacks several important enzymes in nitrate and sulfate metabolism suggesting that it is dependent on the host to provide other sources of reduced nitrogen and sulfur [4••].

One can envision that effectors not only act on plant defence pathways, but also interfere with host metabolic pathways or transporters, redirecting nutrients and changing host metabolism. Bacterial and fungal pathogens manipulate host sugar transport by activating *SWEET* transporter genes during infection. In rice, a bacterial effector of the TALE class binds to the *OsSWEET11* promoter to activate the sugar transporter gene [45•].

Changes in host physiology and metabolism that are possibly manipulated by pathogen effectors have been observed in oomycete-infected plants, for example the repression of photosynthesis and induction of sink status increasing the availability of assimilates for pathogens [46]. Genome-wide expression studies of host responses during infection have, unfortunately, not provided a clear picture of transcriptional changes related to host metabolism. Comparison of a compatible and incompatible (plant is resistant) interaction of Arabidopsis inoculated with *H. arabidopsidis* showed a large overlap in expression changes of defence-associated genes between the treatments. By contrast, only a limited number of 17 compatible-specific genes were identified of which none could be directly linked to metabolism [47], suggesting that pathogen-induced metabolic reprogramming of the host is mainly post-transcriptional.

Conclusion

Recent findings highlight the unique complement of effectors that make the oomycetes such remarkable pathogens. More and more effectors that act inside the host cell are being identified while the debate on the mechanism of their translocation continues. The next step is to unravel their effect on host processes and the molecular mechanisms by which they carry out their virulence function. The discovery of CMPG1 as a host target is an important first step towards understanding the activity of the oomycete effector complement. Major challenges remain the development of robust effector bioassays and gene knockout protocols for oomycetes, in particular for the obligate biotrophs. Emerging studies on host protein-effector interactors and structural data on effectors will be instrumental in elucidating the functions of effectors. Plant-oomycete interactions are reaching a complexity that requires a systems biology approach [48] that, as a bonus, will also provide better insights in the physiology of the plant and the host immune network.

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Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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A study of the highly expanded genome of *Phytophthora infestans*. High transposon activity is identified as a probable cause of expansion and diversification of the effector repertoire of this pathogen. The modularity and diversity of Crinkler C-terminal domains is discussed, as well as the evolutionary mechanisms that may underlie these features. A number of Crinkler C-terminal domains are also shown to induce cell death in *Nicotiona benthamiana*.

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Genome analysis of a broad-range oomycete pathogen. Surprising finds include the absence of RXLR-effectors and the presence of a large family of cadherin genes. A novel family of potential effectors is predicted based on the presence of signal peptides, long flanking non-coding regions and an above average proportion of closely related paralogues.

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Genome analysis of the obligate biotroph *Hyaloperonsospora arabidopsidis*. This study focuses on differences between biotrophic and non-biotrophic oomycetes. *Hyaloperonospora* has reduced numbers of hydrolytic enzymes, PAMP-containing proteins and cell death-inducing effector classes, indicating a more stealthy lifestyle. Furthermore, the absence of genes for flagellum machinery supports the absence of a flagellate zoospore stage in this organism.

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The authors link domains expanded in fungal and oomycete plant pathogens, compared to 58 other eukaryotic species, to the presence of signal peptides and differential regulation during infection, establishing a link to pathogenicity. Furthermore, analysis of the occurrence of combinations of two domains revealed a large repertoire of novel combinations that could contribute to the remarkable characteristics of oomycete pathogens.

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This study adds C14 to the short list of known targets of apoplastic effectors. C14 was found by screening papain-like cysteine proteases for inhibition by *Phytophthora* effectors. The importance of C14 for the host is illustrated by increased susceptibility of C14-silenced plants and evidence of diversifying selection in wild potato species.

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Rcr3 is shown to be targeted by distantly related pathogens, supporting predictions by the guard-hypothesis that host targets are probably important components of basal defence and are targeted by independently evolved effectors. *rcr3* Mutants do indeed show increased susceptibility to *Phytophthora infestans*. In contrast to a known Rcr3-interacting fungal effector, the studied oomycete effector does not trigger the Rcr3-associated defence protein.

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