The problem of how fungal and oomycete avirulence proteins enter plant cells

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Recent advances in cloning avirulence genes from a rust fungus and three oomycete species have provided the novel insight that these eukaryotic plant pathogens deliver small proteins into the host cell cytoplasm where they are recognized by resistance proteins. Anne Rehmany *et al.* have recently identified a potential host-targeting signal in oomycete avirulence proteins from *Hyaloperonospora parasitica, Phytophthora sojae* and *Phytophthora infestans* that might be involved in transporting proteins into the host cell. This signal is surprisingly similar to the host targeting signal used by the malaria pathogen *Plasmodium fulciparum* to target virulence proteins to the mammalian host cell.

Some fungal and oomycete pathogens target avirulence proteins to the plant cytoplasm

It has been known since 1996 [1] that bacterial pathogens of plants secrete effector proteins, including avirulence (Avr) proteins, into the host plant cytoplasm via the type-III secretion system. Given that the corresponding plant R proteins are themselves located in the host cytoplasm, the demonstration that bacterial Avr proteins are delivered into host cells was particularly satisfying. But how are eukaryotic pathogens of plants such as fungi and oomycetes detected by plant cells? The interaction between tomato and the fungus *Cladosporium fulvum*, which is one of the best-studied systems, appears to be entirely extracellular [2]. The fungal avirulence proteins are secreted into the apoplast and recognized by membrane-spanning R proteins similar to extracellular receptors. However, the situation for other eukaryotic pathogens is different. The majority of known resistance proteins to fungal and oomycete pathogens are cytoplasmic nucleotide binding site leucine-rich repeat (NBS-LRR) proteins, just as they are for bacterial pathogens. Thus, the conundrum of how these resistance proteins detect their corresponding fungal or oomycete avirulence proteins has been intriguing. The first cloned pair of R–Avr proteins relevant to this question was the rice Pi-Ta resistance protein, an NBS-LRR protein, and the secreted Avr protein Avr Pi-Ta, from the rice blast fungus *Magnaporthe grisea* [3]. Here, direct interaction between the two proteins was demonstrated in yeast two-hybrid and *in vitro* systems suggesting that this Avr protein also enters the plant cell. Unlike *Magnaporthe*, many other pathogens, such as the rust and mildew fungi, oomycetes including downy mildews, white blister rusts and *Phytophthora* species, form specialized feeding structures called haustoria that penetrate the host plant cell wall but remain separated from the host cytoplasm by a double membrane [4]. Recently, the first *Avr* genes from some of these haustorium-forming eukaryotic pathogens have been cloned and characterized (Table 1) [5–10]. All the *Avr* genes characterized to date encode small proteins with N-terminal secretion signals targeting them to the endoplasmic reticulum secretion pathway of the pathogens; screening directly for pathogen genes encoding haustorially expressed secreted proteins greatly enriches for *Avr* genes [5]. Because gene transfer to these pathogens is not available or is difficult, *Agrobacterium*-mediated or biolistic transient expression analyses of the cloned avirulence genes have been used to confirm indirectly the avirulence functions of the cloned genes by inducing a resistance gene-specific hypersensitive response (HR) in host plants. Expression of truncated forms of these genes that lack the secretion signal also induces a HR [5,6,8–10]. These data have been interpreted as an indication that the avirulence proteins are detected in the host cytoplasm, which is consistent with the observation that corresponding R proteins are of the cytoplasmic NBS-LRR class. In the case of the flax rust fungus, it has been shown that *Avr* gene expression occurs in haustoria [5,6]. Although not yet demonstrated, it seems likely that this will also be the case for oomycetes. So, there are two key questions: what is the function of these Avr proteins (surely not to allow the host to recognize the pathogen) and how do these secreted proteins enter the host cell? Some insight into the second question has now been provided for oomycetes at least [10].

A potential host-targeting signal for oomycete Avr proteins?

An exciting new result from a collaboration of four different laboratories studying the oomycete pathogens *Hyaloperonospora parasitica* and two *Phytophthora* species has shed light on a possible signal that might tag the secreted proteins for uptake by the host cell [10]. These groups have used bioinformatics to recognize a short, conserved amino acid sequence signature, termed the 'RxLR' motif, that occurs within 30 or so residues of the N-terminal signal peptide and is common to the known oomycete avirulence proteins as well as to other secreted proteins of unknown function. This motif consists of the sequence 'RxLRx5-21ddEER' (where R=Arg, x=any

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Table 1. Cloned avirulence genes from haustoria-forming plant pathogens encode small secreted proteins with no similarity to other known proteins

Gene	Pathogen species	Avr protein size[a] (number of amino acids)	Host	Refs
AvrP4	*Melampsora lini*	95	Flax	[5]
AvrM	*Melampsora lini*	314–377	Flax	[5]
AvrP123	*Melampsora lini*	117	Flax	[5]
AvrL567	*Melampsora lini*	150	Flax	[6]
Avr1b-1	*Phytophthora sojae*	138	Soybean	[7]
ATR13	*Hyaloperono-spora parasitica*	154–187	*Arabidopsis*	[8]
ATR1	*Hyaloperono-spora parasitica*	311–321	*Arabidopsis*	[9]
Avr3	*Phytophthora infestans*	147	Potato	[10]

[a]Number of amino acid residues before cleavage of signal sequence.

residue, L=Leu, E=glutamate and d=frequently aspartate), which is related to a protein transport motif, 'RxLxE/Q' (abbreviations as above, Q=glutamine), that occurs in a similar location near the signal peptide in virulence proteins secreted by the malaria pathogen *Plasmodium fulciparum*. After infection, *P. fulciparum* occupies a parasitophorous vacuole within the cytoplasm of the mammalian red blood cell such that host and parasite cytoplasms are separated by two membranes, which is reminiscent of the situation for haustoria in infected plant cells [11,12]. Assays using YFP-tagged proteins expressed in *P. fulciparum* have confirmed the role of the 5-amino acid core motif in targeting these proteins to the cytoplasm of the host red blood cell. However, the transport mechanism for these proteins is not yet defined and it is not clear whether it relies on host- or pathogen-encoded machinery. It seems likely that the RxLR motif is involved in host cell targeting of oomycete Avr proteins, although this function has yet to be demonstrated. So it appears that oomycete avirulence proteins enter the host cell in a two-step process involving signal peptide-mediated secretion followed by host cell uptake mediated by a second signal. Although this is the case for most of the host cell-located malarial proteins, at least one *Plasmodium* protein lacks a signal peptide but has a host-targeting signal [11]. This indicates that another secretion system might be operating and that workers in plant–pathogen systems need to keep an open mind about using an N-terminal secretion signal as an absolute criterion for avirulence protein discovery. The RxLR motif is not detected in flax rust Avr proteins and might be specific for oomycetes [5,6].

A multitude of pathogen proteins targeted to the host plant cytoplasm?

As a result of bio-informatic analysis of the *P. fulciparum* genome, ~400 genes (~8% of the genome) have been recognized that encode proteins carrying the host-targeting sequence, suggesting that hijacking the host cells during malarial disease is genetically complex [11,12]. Are plant–pathogen interactions likely to be similarly complex? Bio-informatics and functional screening of the plant bacterial pathogen *Pseudomonas syringae* have been identified at least 30 proteins that are predicted to travel the type-III secretion pathway [13,14]. A similar number of avirulence genes have been defined by genetic analysis of flax rust [15] and all 16 cloned flax rust resistance genes encode NBS-LRR proteins, suggesting that many if not all these rust Avr proteins enter the host cell [5,6]. Anne Rehmany *et al.* [10] report that >40 *P. infestans* genes encode secreted proteins with the RxLR motif but, given the limited amount of genome sequence that has been analysed to date, this could still be just the tip of the iceberg. Molecular traffic (such as water and nutrients) from the host cell to the haustorium has been known for some time [4] but it is now clear that the plant–haustorial interface is a two-way street. It is thought that eukaryotic plant pathogens form a close association with their plant hosts through haustoria, that they secrete various proteins into the extracellular space between the haustorial and host membrane and that these are taken into the host cell (Figure 1). Recently, a protein of unknown function secreted from the bean rust haustorium has been immunolocalized to the host cytoplasm and nucleus [16]. Specific host-targeting signals might play a role in their uptake into the host cytoplasm. A potential signal has been identified for the oomycete proteins but has not yet been identified for fungal proteins. The mechanism for protein uptake by the host is not known. Further insight might also be gained from studying the uptake of the small (178 amino acids), host genotype-specific protein toxin Tox A, which is secreted into the apoplast of wheat by the necrotrophic fungal pathogen *Pyrenophora tritici-repentis*. Protein uptake and entry to the cytoplasm and chloroplasts of sensitive wheat via a pathogen-independent uptake system encoded by a single host gene has been recently demonstrated [17].

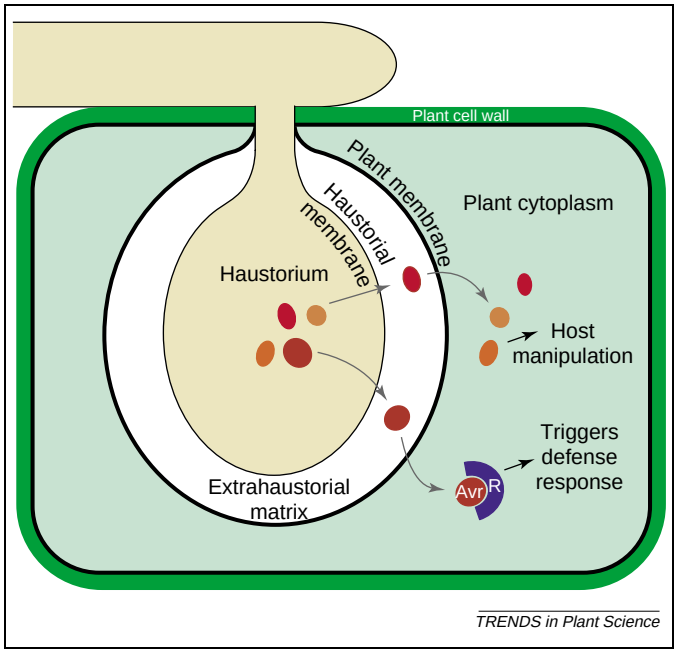

Figure 1. The host plant–pathogen interface. The oomycete or fungal pathogen penetrates the plant cell wall but not the host plasma membrane. Effector proteins (orange and red ovals) are secreted by the pathogen and are postulated to enter the host cytoplasm to alter host metabolism and defence pathways. When recognized by a corresponding resistance protein (R) the effector proteins are referred to as avirulence (Avr) proteins.

Cloning this wheat gene could reveal information about the protein import system that might also be relevant to biotrophic pathogens. The identification of further fungal and oomycete proteins that enter the plant cell, their functions and protein-uptake mechanisms will be a fascinating area for development and for understanding the basis of plant pathogenesis. These insights could provide novel biotech control methods through genetics or targeted chemicals to control this important and destructive class of plant disease-causing organisms.

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Letter

Crosstalk

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Many articles use the term crosstalk to describe general, often indirect influences between signaling pathways that are operationally defined by biochemistry or genetics. Such a general term is popular because it encompasses positive and negative signaling, layered changes in gene expression, and feedback [1]. In other literature, crosstalk is used to describe specific interactions between components of more than one pathway. This occurs between GTPases whereby one depresses or elevates the activity of another by stimulating a GTPase Activating Protein (GAP) or a Guanine nucleotide Exchange Factor (GEF), and proteins exist with both GAP and GEF domains for different GTPases [2]. These general and specific meanings imply that crosstalk acts to balance signal specificity (at one extreme: one physiological output for every input) and signal integration (at the other extreme: one output common to all inputs). Interestingly, both uses of crosstalk are at odds with its earlier definition as '…unwanted signals in a communication channel…caused by transfer of energy from another circuit…' [3]. By this definition, crosstalk occurs when signaling specificity is lost and biological circuits should be designed to minimize it. No matter which definition one prefers, it is reasonable to ask whether the robustness of biological signaling occurs primarily as a consequence of or in spite of crosstalk. Two examples are illustrative.

Bacterial two-component systems are models for how organisms integrate multiple inputs into appropriate responses, for example, chemotaxis mediated by chemoreception of various molecules. Processing of several signals by different two-component proteins happens when one response regulator interacts with multiple sensors, by phosphorelay attenuation by phosphatases, and via transcriptional and post-transcriptional mechanisms. Interaction specificity between cognate pairs of sensor/regulators is mediated via conserved sensor residues near the phosphorylatable histidine, and conserved and hypervariable residues around the phosphorylatable

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