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# Identification of Rust Fungi Avirulence Elicitors

Rust fungi (Basidiomycetes of the order Uredinales) are obligate biotrophs that grow and reproduce only in living plant tissue. There are on the order of 5000 or more species of rust fungi that collectively cause disease on most crops, ornamentals, and many other plants. For example, rusts caused by *Puccinia* species are some of the most important diseases of wheat and other small grain crops worldwide. A new wheat rust epidemic is currently building in East Africa with the appearance of a highly virulent strain of *Puccinia graminis tritici* called Ug99, which is perceived as a threat to global wheat production and has led to the establishment of a Global Rust Initiative (<http://www.globalrust.org/index.html>). Common maize rust caused by *Puccinia sorghi* is a major disease problem of maize, particularly in tropical and subtropical regions such as South Africa and India. The Asian soybean rust caused by *Phakopsora pachyrhizi* has recently spread to Africa and the western hemisphere, including the U.S., and is now a major concern in most of the soybean-growing regions of the world.

Rust fungi have extremely complex life cycles, involving up to five different spore-producing stages. Many rusts are heteroecious, requiring two phylogenetically distinct host plants to complete their life cycle. For example, the wheat rust *Puccinia graminis* alternates between wheat as the primary host and barberry as the alternate host, and *Melampsora epitea* willow-conifer rusts alternate between a coniferous primary host, such as hemlock or tamarack, and a willow alternate host. Some rusts, such as the flax rust *Melampsora lini*, are autoecious and complete their life cycle on a single host plant.

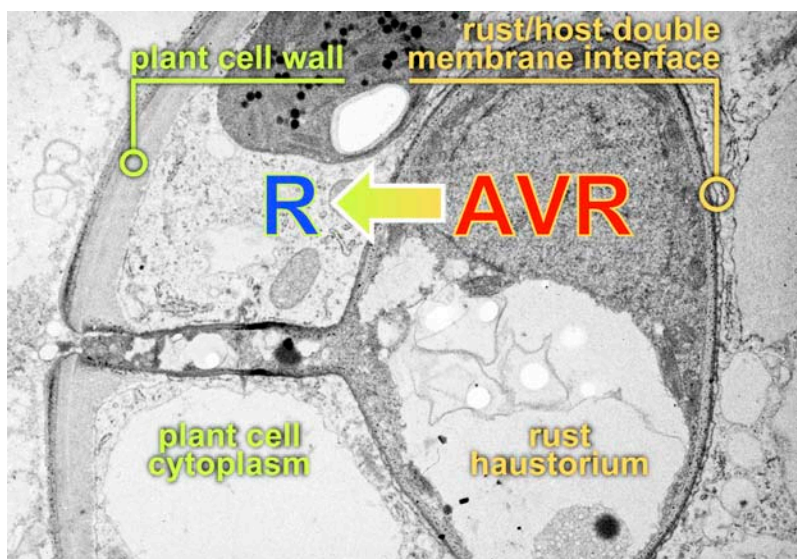
Rust fungi are host specific and will develop compatible or incompatible associations with their host plants in a gene-for-gene manner, depending on the presence or absence of avirulence (*Avr*) genes in the pathogen and corresponding resistance (*R*) genes in the host. The interaction of *M.*

*lini* with flax (*Linum usitatissimum*) has a central place in the history of plant pathology, as studies with this pathosystem led to the development of the gene-for-gene hypothesis of plant-pathogen interactions, one of the most important principles of plant pathology (Flor, 1942, 1955).

During infection of a host plant, rust fungi form haustoria, specialized infection structures that penetrate the plant cell wall and form invaginations in the plasma membrane that are believed to form the major sites of nutrient uptake from the host cell (Hahn and Mendgen, 2001). It is also thought that signals emanating from haustoria suppress host defense responses and facilitate disease in sensitive host plants (Panstruga, 2003; Vogele and Mendgen, 2003) or trigger

a hypersensitive response (HR) leading to disease resistance in resistant hosts (i.e., interaction between *Avr* factors and host *R* gene products; Heath, 1997).

In this issue of *The Plant Cell*, **Catanzariti et al. (pages 243–256)** identify a number of avirulence elicitors (*Avr* proteins) encoded by genes expressed in haustoria of the flax rust *M. lini* (see figure). The previously identified *AvrL567* proteins from flax rust are expressed in haustoria and contain secretory pathway signal peptides, suggesting that they are secreted into the extrahaustorial matrix (Dodds et al., 2004). In addition, all 19 of the flax *R* genes identified to date encode predicted cytoplasmic TIR-NBS-LRR proteins, suggesting that the corresponding rust *Avr* genes encode secreted proteins that somehow gain entry into the



Electron Micrograph of a Stem Rust Haustorium in a Wheat Mesophyll Cell.

The rust haustorium develops after penetration of the host cell wall by a fungal hypha but remains external to the host cell. Host-derived nutrients cross the host and then fungal plasma membranes to enter the haustorium. There is evidence that proteins, including *Avr* proteins, are secreted by the fungus across the haustorial membrane by type I secretion and are then taken up by the plant cell using as yet undefined transport mechanisms. Host resistance results from recognition of *Avr* proteins by host cytoplasmic *R* proteins. In the absence of *R* protein recognition, it is believed that *Avr* proteins are effectors of pathogen virulence. (Figure courtesy of Peter Dodds, Jeff Ellis, and Tony Pryor.)

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host plant cytoplasm. Therefore, the authors searched for haustorially expressed secreted proteins (HESPs) by screening a flax rust haustorium-specific cDNA library for putative secreted peptides.

Among 21 HESPs identified, four cosegregated with the known flax rust avirulence loci *AvrL567*, *AvrM*, *AvrP4*, and *AvrP123*. *AvrL567*, previously cloned by Dodds et al. (2004), encodes a novel small secreted protein that induces an HR dependent on the presence of the cytoplasmic NBS-LRR L5, L6, or L7 resistance proteins in the host plant. Catanzariti et al. focused on characterization of *AvrM* and *AvrP4* and to a lesser extent *AvrP123*. Transient expression of *AvrM*, *AvrP4*, and *AvrP123* induced HR-like responses in flax leaves dependent on the presence of functional corresponding resistance alleles *M*, *P4*, and *P1/P2*, respectively, indicating that the genes encode functional avirulence proteins.

*AvrP123* is a complex locus corresponding to flax resistance genes *P1*, *P2*, and *P3*. The authors identified an HESP corresponding to one gene at this locus, which they named *AvrP123-A*, and genomic DNA gel blot analysis suggested the presence of several additional homologs. The gene was found to encode a small Cys-rich secreted protein that contains a sequence characteristic of Kazal Ser protease inhibitors, suggesting a possible function in pathogenesis of *M. lini* as an inhibitor of host proteases. *AvrP4* was also found to encode a small Cys-rich secreted protein and may have a Cys knot structure similar to that found in some inhibitors of receptors or proteases. *AvrP4* corresponded to a single gene, present in different rust strains as an avirulence (*Avr*) or virulence (*avr*) allele.

*AvrM* was found to be a complex locus that encodes at least six predicted homologous proteins. Five of the sequences segregated with the avirulence allele and were labeled *AvrM-A*, *-B*, *-C*, *-D*, and *-E*, whereas the sixth corresponded to the virulence allele (*avrM*). The *AvrM* proteins showed no similarities to other known proteins or peptide motifs. The predicted *avrM* (virulence) protein was unique among the six in having two in-frame deletions and 10 unique amino acids not shared by the *AvrM* proteins. Analysis of genomic sequence surrounding

the set of *AvrM* genes suggests that they evolved from large duplication events.

Nucleotide variation at both the *AvrM* and *AvrP4* loci showed evidence for diversifying selection acting on these genes. Sequence analysis showed an excess of nonsynonymous (i.e., leading to amino acid changes) over synonymous changes between the virulence allele *avrM* compared with the five *AvrM* avirulence alleles and an excess of nucleotide substitutions in the coding regions of *AvrM* alleles compared with flanking DNA. An excess of nucleotide substitutions was also found between the *AvrP4* gene and the virulent *avrP4* allele compared with that of flanking DNA. These results suggest that positive selection has acted on the divergence of *AvrP4* and *avrP4* alleles and on the accumulation of sequence differences between *AvrM* homologs and the *avrM* allele.

The authors conducted experiments to determine whether the signal peptides of *AvrM* and *AvrP4* function as secretion signals in the plant to direct the protein through the endoplasmic reticulum (ER). They show that addition of the HDEL ER retention signal to the C terminus inhibited the necrotic response induced by the full-length *AvrM-A* protein. Importantly, addition of the chemically similar peptide HDDL, which does not function as an ER retention signal, did not affect *AvrM-A* recognition. These results showed that the predicted signal peptide is functional in plants, and when expressed in plants, the *AvrM* protein is secreted and reenters the cytoplasm from the apoplast. Addition of either HDEL or HDDL signals abolished recognition of *AvrP4* in planta, indicating that the presence of these additional amino acids prevented recognition of this protein irrespective of ER retention. However, truncated *AvrP4* lacking the signal peptide yielded significantly reduced recognition (as judged by the occurrence of a necrotic response) in flax carrying the corresponding resistance *P4* gene, whereas replacement of the predicted *AvrP4* signal peptide with a 44-amino acid plant secretion signal sequence restored recognition, suggesting a requirement for secretion for full recognition of *AvrP4*. It also may be important to acknowledge that *AvrP4* lacking the signal

peptide was still capable of causing a degree of necrosis (although much less than that of the secreted *AvrP4*), indicating that there was some recognition of the non-secreted peptide, and this must have occurred in the host cytoplasm.

In addition, the Cys-rich *AvrP4* contains six Cys residues in the last 28 amino acids that comply with the spacing consensus of inhibitor Cys knot structures, which is similar to the *Cladosporium fulvum* AVR9 protein. Several other Cys-rich avirulence proteins are subject to posttranslational processing, including *C. fulvum* AVR9 (Van den Ackerveken et al., 1993) and *Fusarium oxysporum* SIX1 (Rep et al., 2004). The authors suggest that passage of *AvrP4* through the oxidizing environment of the ER may be required for the formation of disulfide bonds. They further show that mutation of two Cys residues in the last 28 amino acids caused a loss of recognition, as expected if these residues participate in structurally important disulfide bonds.

The work of Catanzariti et al. represents an important contribution to the study of the pathogenicity mechanism of biotrophic parasites in general and rust fungi in particular. It also adds to our understanding of the development of resistance to rust diseases in plants. Taken together, the data strongly suggest the existence of a transport mechanism in the host plasma membrane for secreted *Avr* proteins to gain entry to the host cell. In a recent study of the rust fungi *Uromyces fabae* and *Uromyces striatus*, which infect *Vicia faba* and *Medicago truncatula*, respectively, Kemen et al. (2005) reported the identification of haustorially expressed novel proteins (*Uf*-RTP1p and the homologous *Us*-RPT1p) that localized to the extrahaustorial matrix and entered the host cell cytoplasm by an unknown mechanism, which further supports this hypothesis. Catanzariti et al. discuss two possibilities for this translocation mechanism: a specialized translocation apparatus produced by the rust fungus itself or a host plant transport mechanism. Their experiments with transient expression of the *M. lini* proteins suggest that the secreted proteins are able to enter the host cytoplasm in the absence of the pathogen. In addition,

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the described method of screening fungal haustorium ESTs to search for secreted peptides may be applicable to other biotrophic pathogens, such as other rust species as well as downy and powdery mildews that cause severe diseases on a variety of monocotyledonous and dicotyledonous crop species.

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