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## **Recognition events in gene-for-gene resistance to flax rust**

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Rust fungi are obligate biotrophs that derive their nutritional resources from living host plant cells. During the infection process intercellular hyphae differentiate to produce haustoria, which penetrate the host cell wall and invaginate the plasma membrane. Haustoria are the primary sites for nutrient uptake and host cell communication but are also the site of recognition in resistant plants (Hahn and Mendgen, 2001; Heath, 1997). Other obligate biotrophs, such as the downy mildews (oomycetes) and the powdery mildews (ascomycetes), also produce haustoria although these have probably evolved independently. Hemibiotrophic pathogens, such as the oomycete *Phytophthora*, also form haustoria early in infection, but later induces host cell death and enters a necrotrophic phase.

The flax (*Linum usitatissimum*) and flax rust (*Melampsora lini*) disease system has been an enduring model system in plant disease resistance, having been the basis for Flor's "gene-for-gene" model. In gene-for-gene resistance, the products of host resistance (R) genes determine recognition of pathogen "avirulence" (Avr) gene products to initiate defense responses leading to resistance. One of the hallmarks of this system is the high degree of specificity between corresponding R and Avr genes. Previous isolation of flax resistance genes, including 11 alleles of the *L* locus and representatives of the M, N and P loci, has provided insights into resistance gene specificity. The

recent identification of flax rust Avr proteins has now allowed more detailed analysis of the recognition events that trigger rust resistance.

### **Flax R genes and flax rust Avr genes and their products**

Genetic studies of the interaction between the flax plant and flax rust have identified about 30 flax resistance (R) genes, which occur as series of closely linked or allelic genes at 5 loci, and about 30 corresponding flax rust avirulence (Avr) genes that are mostly dispersed in the flax rust genome. 19 different rust resistance genes have now been cloned from flax, including 11 allelic variants of the *L* locus and representatives of the *M*, *N* and *P* loci (Ellis et al., 1999; Anderson et al., 1997; Dodds et al., 2001a and b). These genes all encode predicted cytosolic resistance proteins of the nucleotide binding site (NBS) and leucine rich repeat (LRR) class, with an N-terminal TIR domain with homology to the Toll and Interleukin-1 Receptor proteins. Domain swap experiments have shown that the LRR domain is the major determinant of specificity in the recognition of the corresponding Avr genes.

Until recently the isolation of avirulence (Avr) genes from biotrophic fungi and oomycetes has been difficult because these organisms cannot be readily cultured or transformed. However, four families of Avr genes, *AvrL567*, *AvrM*, *AvrPI23* and *AvrP4* have now been identified in flax rust (Table 1; Dodds et al., 2004, Catanzariti et al., 2006). The first of these (*AvrL567*) was isolated by a subtractive hybridisation screen for rust genes expressed during infection followed by genetic mapping in a rust family segregating for multiple Avr specificities. The subsequent three were isolated by screening a cDNA library from rust haustoria by ConA-affinity chromatography (Hahn and Mendgen, 1992) for genes encoding secreted proteins. All four Avr gene families encode small secreted proteins that are expressed in haustoria and are apparently translocated into host cells during infection. Evidence for this translocation comes from the observation that transient expression of these Avr proteins as cytoplasmic proteins (ie lacking the signal peptide) in plants can trigger a defense response dependent on the corresponding R genes. This shows that Avr protein recognition occurs inside plant cells, implying that these proteins are translocated during infection. It seems likely that these proteins are part of a larger suite of proteins (probably including the other 20 or so flax rust Avr gene products) that are secreted from rust haustoria and translocated into the plant cytoplasm. These proteins represent a set of host-targetted effector proteins that are presumed to play roles in promoting the infection process. Biotrophic and hemibiotrophic oomycetes have also been recently found to secrete large arrays of effector proteins that are directed into the host cytoplasm during infection (Kamoun et al., 2006). These oomycete proteins are characterised a conserved RxLR

motif that is related to a transport signal responsible for uptake of secreted proteins of the malaria parasite (*Plasmodium falciparum*) across the erythrocyte vacuolar membrane (Hiller et al. 2004; Marti et al., 2004, Bhattacharjee et al., 2006). Thus there appears to be a conserved translocation mechanism used by these distantly related plant and animal pathogens. However no such motif occurs in the flax rust Avr proteins, which probably utilize a different uptake mechanism. Initial experimental data indicate the likely route of uptake is via a host encoded system rather than a specialized rust secretory system analogous to the type three secretion system used by bacterial pathogens. For example, transient expression of the AvrM protein with or without the signal peptide induces an *M* gene specific HR, but addition of the HDEL endoplasmic retention signal prevents recognition of the secreted but not the cytoplasmic version (Catanzariti et al 2006). This is consistent with recognition of the secreted form by the cytoplasmic M protein *after* secretion and re-entry into the plant cell.

### The molecular basis of Avr protein recognition and gene-for-gene specificity

The co-localization of Avr and R proteins in the flax cytoplasm and the genetics of the gene-for-gene interactions are consistent with direct interaction between these proteins. This hypothesis has been investigated experimentally using the yeast two hybrid system to detect R-Avr protein interactions. In these experiments the full length L5, L6 alleles and a chimeric construct L6L11RV that differs from L6 by 11 amino acid

Table 1. Characteristics of cloned avirulence/virulence genes and their protein products from flax rust, <i>Melampsora lini</i>				
Locus	Gene copies per haplotype	Variant forms	Predicted size mature protein (amino acids)	Protein features
<i>AvrL567</i>	Various 1, 2, 3, or 4	12	127	Novel – no data base matches Highly variable (25% of amino acids polymorphic)
<i>AvrM</i>	Various 1, 5 (+?)	6	184 to 349	Novel – no data base matches Expressed in germ tubes as well as haustoria.
<i>AvrP4</i>	1	3	67	Novel – no data base matches Six cysteine residues – potential to form cysteine knot structure Polymorphisms occur in 22 aa C-terminal cys-rich region.
<i>AvrP</i> <i>AvrP123</i>	2 (1 a pseudo-gene)	6	89 to 94	Novel – no data base matches 10 cysteine residues – conform to consensus spacing of Kazal family of protease inhibitors.

differences derived from L11 in the 3 C-terminal LRR units, and 12 AvrL567 variants (AvrL567-A to AvrL567-L) were co-expressed in yeast two hybrid assays. Protein-protein interactions were detected in yeast for the same combinations of L and AvrL567 genes as induced HR in transient expression assays in planta (Dodds et al 2006). The close correspondence between the detection of a protein interaction in yeast and the induction of HR in planta indicates that direct R-Avr protein interaction is the basis for recognition specificity. For example, L6 but not L5 interacts with AvrL567-D in yeast, and co-expression of L6 but not L5 with AvrL567-D induces HR in planta. Furthermore, the L6L11RV chimera interacts with only AvrL567-J in yeast and again induces HR with only this Avr gene in planta. The observation that L6L11RV and L6 differ only in the last 3 LRR units indicates that both the resistance and interaction specificities are controlled by the LRR domain. No interactions were detected in yeast between the resistance proteins and the proteins encoded by the virulence alleles that do not induce HR in flax lines.

Mutation in the P-loop ATP binding motif in the NBS domain of L6 eliminated both the yeast two hybrid interaction and HR response in plants and so while the LRR is clearly the determinant of specificity, the results suggest that ATP or ADP bound to the NBS domain is required for a protein conformation capable of binding Avr proteins. Yeast two hybrid experiments with N- and C-terminal deletions of L6 have shown that the TIR domain is not necessary for R-Avr interactions and that the minimum interacting deletions include both NBS and LRR domains (PN Dodds, unpublished data). These results are similar to those reported by Ueda et al. (2006) for the interaction between the TIR-NBS-LRR N protein of tobacco and the p50 fragment of the TMV replicase protein, the Avr protein of TMV. In this system direct interaction between N-p50 was detected in yeast and in vitro between purified proteins, depended on the binding of ATP to the N protein, and the minimum component of N for interaction was the NBS-LRR fragment.

In the flax system, the observation of direct interaction between L5 and L6 proteins and corresponding Avr proteins has now been extended to M and AvrM (PN Dodds, unpublished results). However, whereas M is approximately 80% identical to L5 and L6, AvrL567 and AvrM proteins are unrelated. Similarly, while L6 and L11 differ by only 32 LRR polymorphisms, their corresponding Avr proteins are also apparently unrelated. In addition, all the other distinct L alleles interact with genetically independent avirulence genes and these are not sufficiently related in DNA sequence to be detected by AvrL567 DNA probes. If as seems likely, all these R proteins directly interact with their corresponding Avr proteins, the picture that is emerging is that NBS-LRR proteins can interact with diverse ligands and that the LRR region is highly flexible in an evolutionary sense with the

capacity to recognize by direct interaction diverse pathogen ligands when coupled with the NBS domain.

### **Co-evolution of *Avr* and *R* genes in the flax rust system**

Recognition by direct interaction has led to a high level of sequence diversity in the rust *Avr* genes as a consequence of strong diversifying selection to escape recognition and host resistance. The *AvrL567* genes are highly variable, with 12 different sequence variants (A-L) found in six rust strains of diverse origin. The 127 amino acid sequence of the mature *AvrL567* proteins contains 35 polymorphic sites, with nine sites showing multiple polymorphisms. These variants have arisen through positive selection, as indicated by the excess of non-synonymous nucleotide substitutions over synonymous changes in their coding sequences. This suggests that there has been a co-evolutionary “arms race” between the corresponding *Avr* and *R* genes in this system. Evidence that the diversification of the *AvrL567* genes is driven by *R* gene-mediated selection comes from the observation that the sequence differences between the *AvrL567* proteins lead to differences in recognition specificity by the corresponding L5, L6 and L7 resistance proteins.

The structures of *AvrL567*-A and -D have been determined by X-ray crystallography (Wang et al, in preparation) and structural modeling indicates that avirulence and virulence variants of this protein have very similar structures and physical properties. The polymorphic residues map to the surface of the protein and polymorphisms in residues associated with recognition differences for the *R* proteins lead to significant changes in surface chemical properties. Analysis of single and multiple amino acid substitutions in *AvrL567* proteins has confirmed the role of individual residues in conferring differences in recognition, but also suggest that the specificity results from the cumulative effects of multiple amino acid contacts.

The fact that naturally occurring virulence forms are expressed and encode products highly related to the avirulence variants suggests that there has been selection for *Avr* variants that escape detection by *R* proteins but retain a selective value for the pathogen, most likely through a virulence effector function. *AvrL567* proteins show no similarity to any known or predicted proteins in current data bases and do not contain any known functional motifs, so the identification of their postulated virulence function is an important target of continuing research. Transgenic flax expressing the rust avirulence genes show no obvious phenotype in the absence of the corresponding resistance gene, and are not compromised in their expression

of resistance to otherwise avirulent rust strains, which could have indicated a suppression of defense activity.

Diversifying selection is also evident in the other flax rust Avr genes, and most particularly the *AvrP123* gene, which like *AvrL567*, encodes an array of allelic variants with diverse recognition specificities for the corresponding *P*, *P1*, *P2* and *P3* resistance genes. Co-expression of the *AvrP123* alleles with the *P* or *P2* resistance genes in tobacco shows a conservation of the recognition and HR induction in this heterologous host, which is also consistent with a direct recognition event that does not require conservation of other host recognition factors. Thus it seems likely that direct R-Avr protein recognition prevails in this disease system, which contrasts with *Arabidopsis*-*Pseudomonas* disease resistance interactions. Part of the evolutionary explanation for this discrepancy may lie in the obligate parasitic and narrow host range characteristics of flax rust compared to bacterial pathogens. Mechanistically, the rust effectors may influence host target proteins through binding interactions rather than enzymatic modifications that can be detected indirectly.

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