

# Flax Rust Resistance Gene Specificity is Based on Direct Resistance-Avirulence Protein Interactions

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## Key Words

disease resistance genes, flax rust resistance, avirulence genes,  
effector proteins, rust fungi

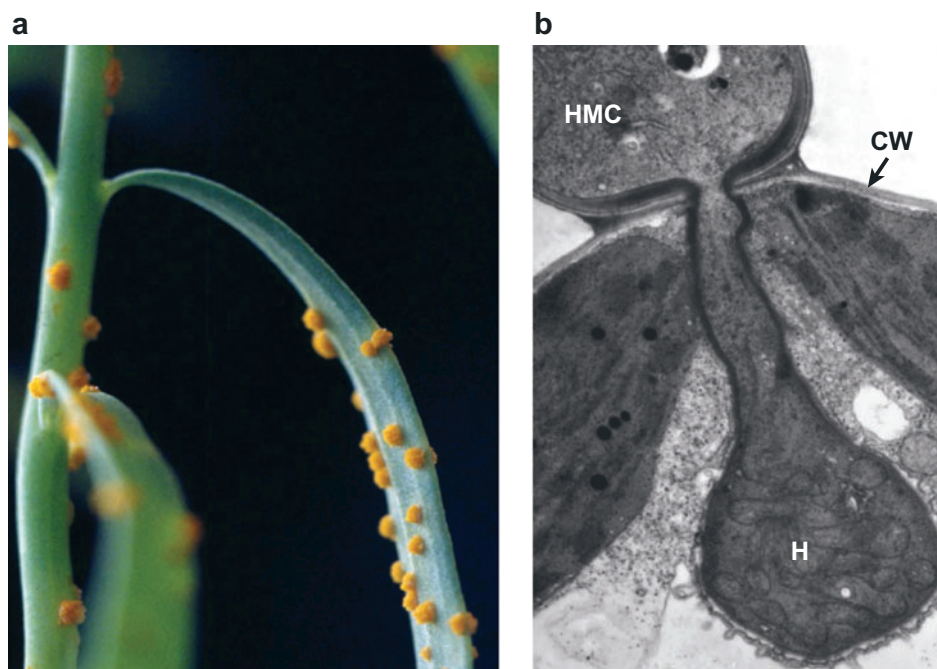
## Abstract

Genetic studies of the flax-flax rust interaction led to the formulation of the gene-for-gene hypothesis and identified resistance genes (R) in the host plant and pathogenicity genes, including avirulence (Avr) and inhibitor of avirulence genes (I), in the rust pathogen. R genes have now been cloned from four of the five loci in flax and all encode proteins of the Toll, Interleukin-1 receptor, R gene-nucleotide binding site-leucine-rich repeat (TIR-NBS-LRR) class. Avr genes have been cloned from four loci in flax rust and encode small secreted proteins with no between locus similarity and no close homologs in current data bases. It is postulated that Avr proteins enter the host cell, have virulence effector functions, and in resistant host genotypes, are recognized by direct and specific interaction with host R proteins, leading to activation of rust resistance defense responses. Direct interaction between R and Avr proteins is the basis of gene-for-gene specificity in the flax-flax rust system and both R and Avr genes have the signatures of diversifying selection, suggesting the existence of a coevolutionary arms race between the host plant and its obligate rust pathogen.

## THE GENETICS OF RESISTANCE/SUSCEPTIBILITY IN FLAX AND AVIRULENCE/VIRULENCE IN FLAX RUST

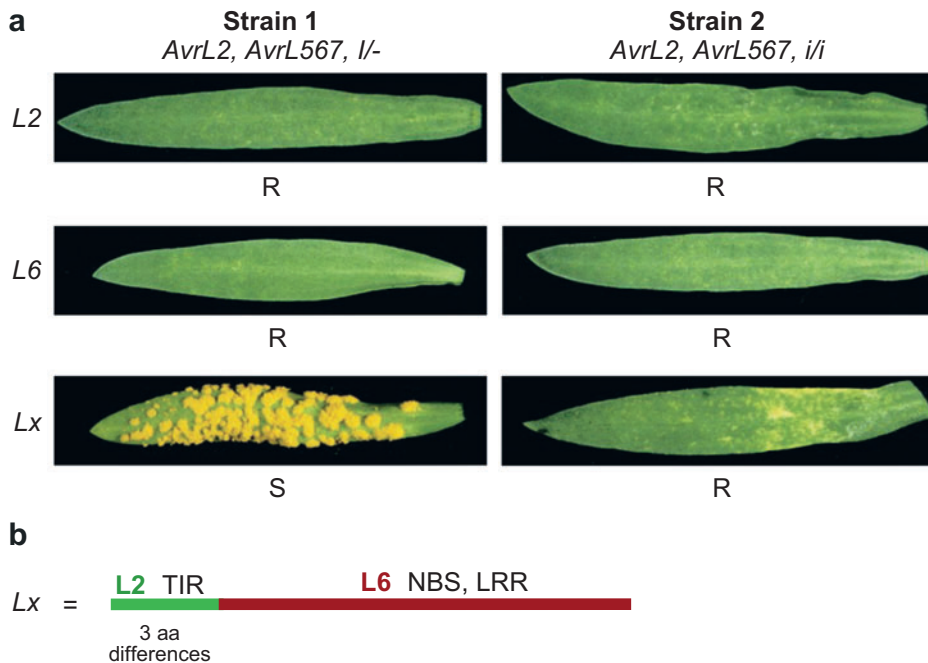
*Melampsora lini* is the rust fungus that infects flax, linseed, and linola crops (*Linum usitatissimum*) and related wild species in the genus *Linum*, giving rise to flax rust disease (Figure 1). Extensive variation for resistance and susceptibility to rust infection occurs in the *Linum* gene pool and simultaneously, extensive variation for pathogenicity occurs in the rust gene pool, with isolates being virulent or avirulent depending on the host genotype under infection. The classical genetic analy-

sis of this interaction carried out by Flor (15) and others (17, 18, 23) has identified three critical types of genes that determine whether the outcome of the plant-fungus interaction is compatible (leading to disease) or incompatible (no disease, or much limited fungal reproduction), namely dominant Resistance (R) genes in flax and dominant Avirulence (Avr) genes and dominant *Inhibitor of avirulence* (I) (17, 18, 23) genes in flax rust (Figure 2). Flax R genes express resistance to (recognize) some but not all flax rust isolates, and different patterns of resistance and susceptibility to isolates of the rust distinguish different resistance gene specificities. Genetic analysis has shown



**Figure 1**

Infection of a flax plant with flax rust. (a) Flax rust urediniospore pustules on the leaves and stem of a rust-susceptible flax plant about 14 days after infection. Urediniospores are the dikaryotic propagules of the flax rust fungus that give rise to cycles of rust infection during the host growing season. Because they are derived from an asexual process they are clones of the infecting rust strain and can be stored under vacuum for 20 to 30 years to enable maintenance of rust genotypes for genetic and molecular analysis of host resistance and rust avirulence genes. (b) Electron micrograph of a section of a flax leaf after establishment of a compatible flax rust urediniospore infection. After germination of the spore on the flax leaf surface and penetration of the leaf interior via a stomate, the rust has formed a haustorial mother cell (HMC) adjacent to the cell wall (CW) of a host mesophyll cell, penetrated the cell wall and formed an haustorium (H). Micrograph from (15a, figure 10) reprinted with permission from Springer-Verlag.



**Figure 2**

The effect of the flax rust *Inhibitor of avirulence* gene (*I*) on flax rust virulence. (a) Two flax rust strains were used to infect flax plants that were homozygous for resistance genes *L2*, *L6*, or *Lx* and leaves were photographed at about 14 days post infection. Rust strain 1 carries *AvrL2* and *AvrL6* and the dominant allele of *I*. Strain 2 carries the same *Avr* genes and is homozygous for the recessive *i* allele. The presence of *I* in strain 1 inhibits the recognition of *AvrL567* by *Lx* but not by *L6*. *I* does not affect *L2-AvrL2* recognition. (From Reference 25 and reprinted with permission from ASPB). R = resistant; S = susceptible. (b) The *Lx* gene is a recombinant between the *L2* and *L6* alleles, encodes a chimeric resistance protein with the TIR region from *L2* and NBS and LRR regions of *L6*, and expresses a specificity identical to the naturally occurring *L7* allele, except for a more complete (no sporulation) resistance. It differs from *L6* by 3 TIR region amino acid polymorphisms derived from the *L2* sequence.

that an *R* gene provides resistance to flax rust isolates carrying a corresponding *Avr* gene but not to isolates lacking the gene: the classical “gene-for-gene” interaction. In some interactions the presence of an *I* gene in the rust modifies the outcome of a specific *R-Avr* gene interaction from resistance to susceptibility. The gene-for-gene interactions in flax-rust and other plant-pathogen interactions gave rise to the hypothesis that *R* genes encode specific receptors for the direct or indirect products of the pathogen *Avr* genes. Testing this proposal and elucidation of the molecular basis of specificity of gene-for-gene interactions has now progressed significantly as a result of

the isolation of several corresponding *R* and *Avr* genes from flax and its rust pathogen. The third player, the *I* gene, is yet to be cloned.

## FLAX R GENES AND THEIR PRODUCTS

In flax 31 specificities for rust resistance map at 5 loci designated *K*, *L*, *M*, *N*, and *P*, which encode 2, 13, 7, 3, and 6 specificities, respectively (16). *R* genes have been cloned from the *L*, *M*, *N*, and *P* loci and all of these encode resistance proteins of the TIR-NBS-LRR class (2, 9, 10, 13, 22). Genes at the *L* and *M* loci are

closely related in nucleotide sequence (>80% identity) and are probably homoeologs in the ancient tetraploid flax genome. The *L* and *M* genes share only low sequence identity with the *N* and *P* genes, which themselves also share very low sequence identity. Thus the cloned flax R genes belong to three distinct sequence lineages. Restriction Fragment Length Polymorphism (RFLP) mapping with both *N* and *P* gene probes detects related genes at unlinked loci that may also be homoeologous loci, one of which could include the as-yet unmapped and uncloned *K* rust resistance locus.

Gene cloning has confirmed that resistance specificities at the *L* locus are encoded by alternative alleles of a single gene, which made cloning the 12 described specificities at the locus (*L*, *L1*, *L2*, *L3*, *L4*, *L5*, *L6*, *L7*, *L8*, *L9*, *L10*, and *L11*) straightforward (13). The sequences of *L3* and *L10* have identical coding regions, and *L4* encodes a single amino acid difference in the NBS region, raising a question about the original differentiation of these three specificities. Gene cloning has also confirmed that the *M* locus structure is different from *L*; approximately 15 paralogues of *M* occur at the complex locus, only one of which encodes the *M* specificity, indicating that genome-specific duplication events have occurred at this homoeolocus (2). Each of the seven *M* locus specificities (*M*, *M1*, —*M6*) occurs in a different haplotype and therefore their cloning is less straightforward as it requires identification of the active member(s) at each complex either by mutagenesis or by cloning and testing all expressed candidates from each haplotype for resistance specificity in transgenic flax.

The *N* and *P* loci also consist of several closely related linked genes (9, 10). The *P2* gene product differs from the *L*, *M*, and *N* proteins by the presence of an additional domain of 150 amino acids C-terminal to the LRR region, called the C-terminal Non-LRR domain (CNL), which is related to sequences in several R proteins in other plant species, including the TMV resistance protein *N* in

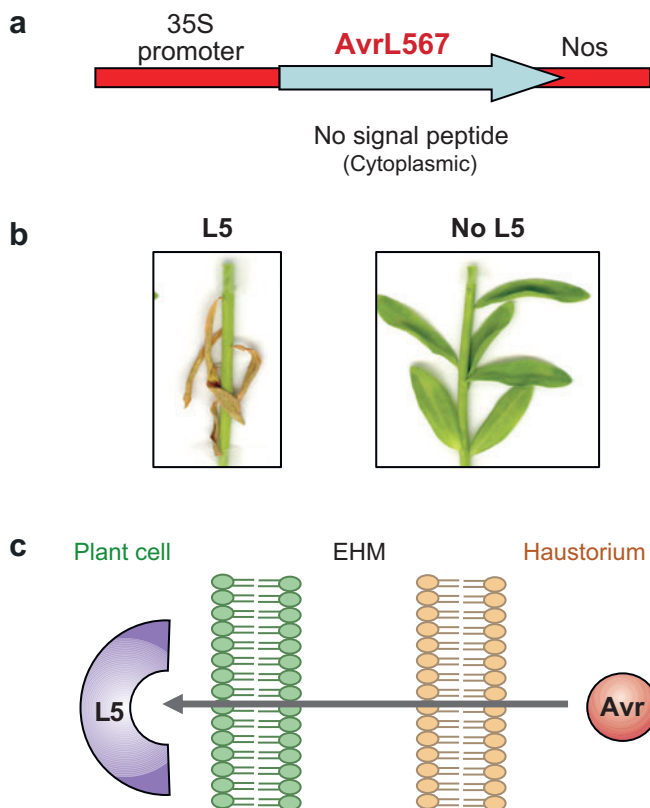
tobacco and RPS4 in *Arabidopsis*. In fact, examination of the *Arabidopsis* genome sequence using the BLAST P searches revealed that 45% of the 83 TIR-NBS-LRR proteins (28) contain a related CNL domain, thus defining two subclasses within the TIR-NBS-LRR class (10). The function of this domain has not been revealed although several mutations in this region in *P2* cause loss of resistance.

The subcellular locations of R proteins, RPM1, RPS2, and Mla1, members of the CC-NBS-LRR class, and RPP1, a TIR-NBS-LRR protein, have been determined experimentally and are intracellular proteins (3–5, 33). The flax rust TIR-NBS-LRR proteins are also predicted to be intracellular. The proteins contain no membrane-spanning regions, and *N* and *P* proteins do not have predicted N-terminal signal peptides. Like the RPP1 protein from *Arabidopsis*, which contains a hydrophobic N terminus that directs the cytoplasmic protein to associate with the ER and/or Golgi membranes (33), the *L* and *M* genes encode hydrophobic N termini that are weakly predicted to be a signal peptide but preliminary data using reporter gene fusions show that this sequence is likely to function as a membrane anchor, suggesting that the *L* and *M* proteins are also intracellular.

## FLAX RUST AVR GENES AND THEIR PRODUCTS

Approximately 30 *Avr* genes have been identified in flax rust by genetic analysis. In contrast to the genetic clustering of host *R* genes, *Avr* genes, with a few exceptions, are not genetically linked. The exceptions are the *AvrL3*, *AvrL4*, and *AvrL10* cluster (which may be a single avirulence specificity given that sequence data suggest *L3*, *L4*, and *L10* are the same resistance specificity), the *AvrL5*, *AvrL6*, and *AvrL7* cluster, and *AvrM1* and *AvrM4* cluster where genes at each cluster cosegregate in genetic crosses. *AvrP*, *AvrP1*, *AvrP2*, and *AvrP3* are also tightly linked with recombinants recovered at approximately 0.2% of test-cross progeny (23, 24).

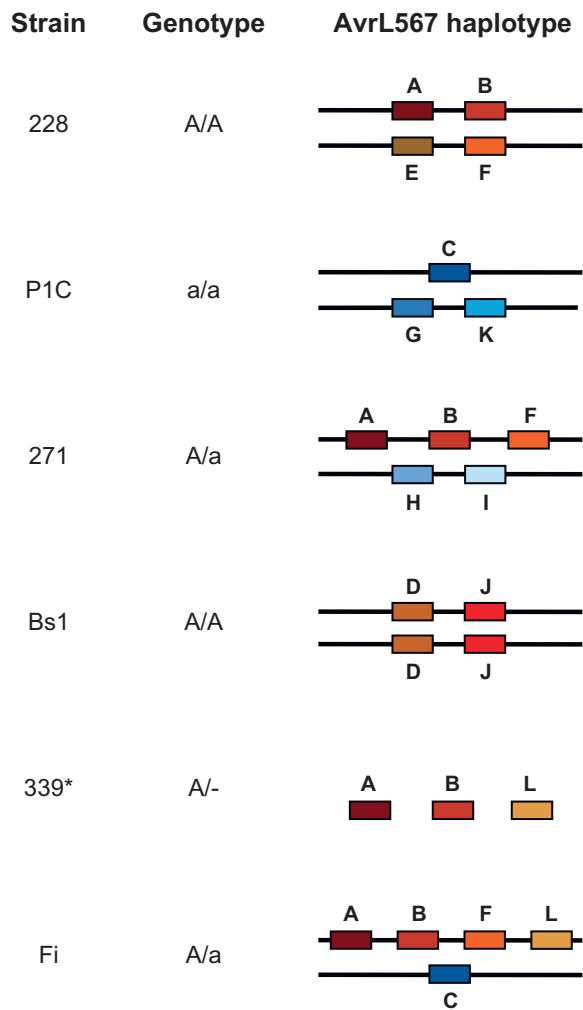
Flax rust *Avr* genes have been cloned from four loci by mapping of candidate genes (6–8). Each locus carries from one to five closely related *Avr* genes and in contrast to the corresponding *R* genes, there is no inter-locus sequence similarity between the four *Avr* gene families. Initially, the technique of suppressive subtractive hybridization was used to identify rust genes expressed during infection. These were then used as DNA probes to detect RFLPs segregating in a flax rust mapping family in which 16 *Avr* genes segregate. One probe detected a locus that cosegregated with the *AvrL5*, *AvrL6*, and *AvrL7* cluster, but significantly, with none of the other unlinked *Avr* loci corresponding to other *L* alleles or *M*, *N*, or *P* genes. Genomic clones of the gene were isolated and, in the absence of a flax rust transformation method, the cloned rust gene was tested for avirulence function by both transient and stable expression in flax lines with different rust resistance genotypes. Transient assays involved infiltration of flax leaves with *Agrobacterium* strains capable of delivering the candidate *Avr* gene expressed with a plant promoter to host cells (Figure 3). Leaf HR (hypersensitive cell death response) in flax lines that carried *L5*, *L6*, or *L7* resistance genes, but not in lines without these resistance genes, demonstrated avirulence function. Similarly, when *AvrL567* genes were expressed in transgenic flax without an effective *R* gene and transgenics crossed to flax lines carrying the corresponding *R* genes, *R* gene dependent seed death or stunted seedling phenotypes arose in progeny. Because the *Avr* gene induced HR in *L5*, *L6*, and *L7* (but not in other *L*) genotypes it was renamed *AvrL567* to reflect these overlapping specificities. DNA gel blot analysis and gene cloning revealed that several copies of the *AvrL567* gene occur in most rust strains. Sequence analysis of genes cloned from 6 rust strains identified 12 variant forms (*AvrL567-A*, *-B*, *-*, *-K*) of the gene, which occur in haplotypes carrying one, two, three, or four copies in the 6 analyzed rust strains (Figure 4). Of these, 7 variants have avirulence activity (induce HR) in transient



**Figure 3**

The *AvrL567* gene product is recognized in plant cells by *L5*. (a) The flax rust gene variant *AvrL567-A* without the 5' sequence encoding the protein secretion signal was modified for plant expression using the CaMV 35S promoter and *nopaline synthase* (*Nos*) 3' mRNA processing sequence and cloned for *Agrobacterium*-mediated transient plant transformation. (b) Transient expression of *AvrL567-A* induced a necrotic response in flax leaves of the *L5* genotype but not in near-isogenic leaves lacking *L5*, *L6* or *L7*. (c) Recognition of a nonsecreted form of *AvrL567-A* within flax cells suggests this protein is transported to plant cells during rust infection. The *AvrL567-A* protein would be secreted across the rust haustorial membrane by the endomembrane secretory pathway into the space between the haustorial cell wall and invaginated host cell plasmamembrane (extrahaustorial matrix, EHM). The secreted protein would then be transported across the plant membrane by an unidentified mechanism and recognized by direct interaction with the cytoplasmically localized *L5* protein.

expression analysis in flax plants carrying *L5*, *L6*, or *L7* genes, whereas 5 virulence variants, do not induce HR. [Throughout this review we refer to variant forms of avirulence genes that are not recognized by known *R* genes as “virulence” genes/alleles/variants. Although a virulence function is postulated (see below)



**Figure 4**

Haplotype structures (gene orders arbitrary) of *AvrL567* genes in six heterokaryotic flax rust strains. The avirulence genotype (A = avirulence, a = virulence, - = unknown) was determined by infection analysis of self or outcross progeny on flax genotypes with *L5*, *L6*, or *L7* resistance genes. (\* no self progeny available for strain 339) *AvrL567* homologs were PCR amplified from parental strains and progeny and sequenced. Avr function was tested by transient expression analysis as described in **Figure 3**. Each haplotype carries from 1 to 4 *AvrL567* genes with >90% DNA sequence identity. Avirulence variants are represented in shades of red to orange and virulence variants in blue shades.

no direct evidence is available yet that either avirulence or virulence forms actively enhance rust virulence. However, virulence forms do enhance disease, at least in a passive sense, because they allow the rust to escape detection by a specific host R gene.] Some of the

avirulence variants can be distinguished based on the specificity of their interactions with *L5*, *L6*, and *L7* plants in transient assays. For example, whereas *AvrL567-A* induces HR on *L5*, *L6*, and *L7* plants, *AvrL567-D* induces HR on *L6* and *L7* but not *L5* (**Figure 5**).

*AvrL567* mRNA was detected in rust-infected leaves and in purified rust haustoria, the specialized feeding structures of these fungi that make the closest contact with host mesophyll cells, but not in ungerminated spores or spores germinated on water in the absence of the host. The 12 *AvrL567* gene variants all encode 150-amino acid preproteins with a conserved N-terminal secretory sequence and predicted mature peptides of 127 residues and so these proteins are probably secreted from the rust. The 12 proteins are highly variable with 25% of the residues having one or more polymorphisms (**Figure 5**), and analysis of the gene sequences indicated that they have undergone selection for diversity (7, 8). Transient and stable expression in flax of *AvrL567* without the signal peptide gave rise to R gene-dependent recognition, indicating that the Avr proteins are recognized inside host cells. This implies that the Avr proteins are secreted from the rust haustoria during infection and enter the host cell (**Figure 3**). The structures of *AvrL567-A* and *-D* have been determined by X-ray crystallography, and structural modeling indicates that avirulence and virulence variants have very similar physical properties with variable amino acids at the surface of the protein (our unpublished data). The fact that naturally occurring virulence forms are expressed and encode products highly related to the avirulence variants and that the variable sites are surface exposed 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

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				<b>Necrotic reaction</b>			
				<b>L5</b>	<b>L6</b>	<b>L7</b>	<b>L6L11</b>
{	Avr	B	H..S.I.K..D...D..A.....C...E....	+	+/-	-	-
		A	HR...I.K.....PK..S.AQQ.NA..EGFE....	++	+	+/-	-
		F	HR...I.K..D...PK....AQH.NAS....E....	++	+	+/-	-
		L	H..S.V.K.....PK....AQ.SHA.....E....	++	+	+/-	-
		J	...S.I.N.....S.....C.....	++	+	+/-	+
		E	H..S...N.....S.....C.....	-	+/-	-	-
		D	.....N....IPK.L..AQH.HA....F.....	-	++	+	-
{	avr	C	.R.....KDL....SIA.....IC.....E..	-	-	-	-
		G	N...T.....D...DQ.A.....F.D...	-	-	-	-
		H	....K....D...DQ.A.....F.D.H.	-	-	-	-
		I	....T.R.T.....S.....C...D...	-	-	-	-
		K	N.T.T.....IPKY...AQH.NA....L.D..N	-	-	-	-

**Figure 5**

Amino acid variation between 12 AvrL567 variants is associated with differences in recognition specificity by L5, L6, L7, and L6L11 [*L6L11R* an in vitro chimera of *L6* and *L11* genes, (12)]. Polymorphic residues in the 12 AvrL567 variants are shown on the left panel, with the consensus residue for each position (numbered vertically) indicated above the alignment. A ‘•’ indicates identity with the consensus. The right panel shows the reactions (+ = necrosis, +/- = weak necrosis, - = no necrosis) of the 4 flax resistance genotypes to Avr genes expressed transiently in flax leaves.

important target of continuing research. Transgenic flax expressing the rust avirulence genes show no visual phenotype in the absence of the corresponding resistance gene, and are not compromised in their expression of resistance to otherwise avirulent rust strains (for instance, resistance mediated by *L9* to rusts carrying *AvrL9*), which would indicate a suppression of host defense activity.

*Avr* genes corresponding to other flax R genes were cloned from a library of rust genes made from RNA extracted from isolated haustoria (6). Approximately 800 cDNA clones were sequenced and screened for sequences encoding secreted proteins based on the presence of predicted signal peptides. Among 20 candidates, 3 genes were identified that cosegregated with and, in transient Agrobacterium expression assays in flax leaves, expressed the avirulence phenotypes of *AvrM*, *AvrP4*, and the complex locus containing the *AvrP*, *AvrP1*, *AvrP2*, and *AvrP3* avirulence

gene cluster. This indicates that this approach may be generally useful for enriching cloned rust *Avr* gene candidates. However, given that recently cloned *Avr* genes from the barley powdery mildew pathogen do not encode an N-terminal secretion sequence and are recognized by R proteins located in the host plant cytoplasm, the use of the secretion signal as a criterion for selecting *Avr* candidates could lead to some potential candidates being discarded in this screen (30). In contrast to the host resistance proteins, the flax rust avirulence gene products share no sequence similarity between the four cloned loci, and DNA probes from these clones do not detect any of the remaining uncloned flax rust *Avr* genes.

At the *AvrM* locus in rust strain CH5 there are at least 5 paralogs, *AvrM-A*, *-B*, *-C*, *-D*, and *-E*, with each gene separated by at least 10 kb. A single virulence gene *avrM*, not recognized by any known flax R gene, occurs at the alternative allele of this locus. *AvrM* transcripts

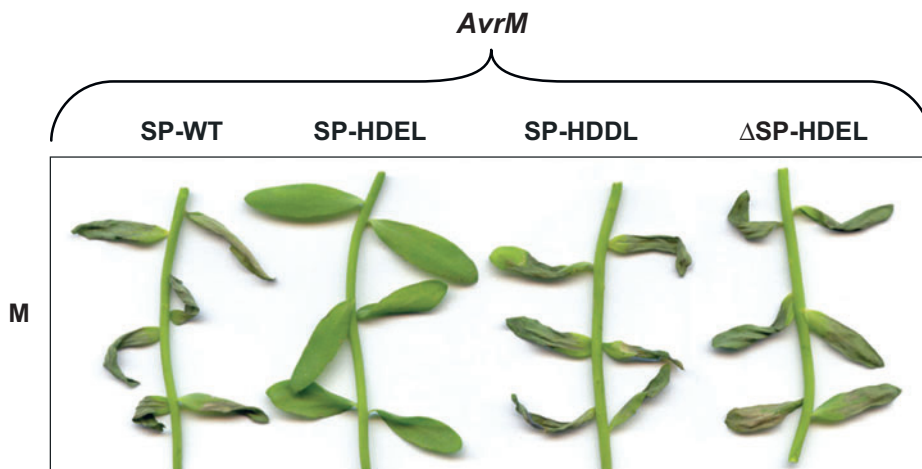
are not detected in ungerminated spores, are expressed in haustoria and, in contrast to *AvrL567*, are expressed in spores germinated in the absence of the host. AvrM proteins are not related to any proteins in current data bases (except a Genbank EST isolated from poplar leaves that may well derive from infection with poplar rust, which is in the same genus as flax rust) including *AvrL567*, and are considerably larger than *AvrL567*, ranging in size from 212 to 377 residues. The size variation of AvrM proteins is a result of DNA insertions and deletions or polymorphisms in the location of stop codons. Transient expression of these genes in flax indicated that AvrM-A, -B, -C, and -D are recognized by the M resistance protein but the truncated AvrM-E is not.

The *AvrP4* locus in strain CH5 contains a single gene with alternative virulence and avirulence alleles that both encode a secreted protein with a predicted mature size of 67 amino acids with no sequence similarity to any known proteins. A third variant was isolated from rust strain WA. In the C-terminal 22 residues there are 6 cysteine residues with the potential to form a cysteine knot structure. Sequence comparison of the two avirulence proteins and one virulence form indicates that these proteins are also highly polymorphic, with the variation concentrated in the C-terminal 22 residues between the conserved cysteine residues. The corresponding *P4* resistance specificity has not been cloned yet.

The fourth cloned *Avr* locus in flax rust, like *AvrL567*, encodes a complex set of overlapping avirulence specificities corresponding to the *P*, *P1*, *P2*, and *P3* resistance genes (6). In our rust mapping family, one allele of this locus encodes avirulence on *P*, whereas the alternative allele encodes avirulence on *P1*, *P2*, and *P3*. Each haplotype contains one functional copy of the *Avr* gene, designated *AvrP* and *AvrP123*, which encodes these recognition specificities when transiently expressed in flax plants. The predicted mature AvrP and AvrP123 proteins contain 89 and 94 amino acids, respectively, and contain 10 cysteine

residues that conform to the consensus spacing of the kazal family of protease inhibitors, providing a clue to the function of these proteins. The two variants differ by 36 amino acid polymorphisms, with a large excess of nonsynonymous nucleotide substitutions, again indicating diversifying selection acting on this locus. Extensive sequence variation in these genes is also seen in other rust strains and is associated with further variation in recognition specificity by the corresponding *P* locus resistance genes (unpublished results).

The presence of signal peptides in the identified flax rust Avr proteins and the timing of their expression during infection indicates that they are secreted by the rust, probably into the extrahaustorial space between the haustorial cell wall and the host plasma membrane. Current experimental data indicate the route of uptake may be via a host-encoded system and not a specialized rust secretory system analogous to the type three secretion system used by bacterial pathogens (6). For example, transient expression in flax leaves of *AvrM* transgene constructs that encode proteins with and without the signal peptide induces an *M* gene-specific HR (**Figure 6**). This is consistent with recognition of the nonsecreted form by the cytoplasmically located M protein, and with secretion, re-entry, and recognition of the secreted form of the protein. In the same transient assay, addition of the HDEL endoplasmic retention signal to the secreted form of AvrM prevents host cell HR, whereas addition of the nonfunctional HDDL form of the signal induces HR. Cytoplasmic expression of AvrM-HDEL (no signal peptide) gives rise to HR in these assays, which shows that the HDEL motif does not interfere with recognition or protein stability. These data imply that in this transgenic system the AvrM protein is secreted via an endoplasmic reticulum route, that the HDEL signal causes retention of the protein in the ER where it cannot be detected by the cytoplasmic M protein, that deletion of the signal peptide prevents the functional HDEL form from entering the ER and allows its recognition



**Figure 6**

A host-encoded uptake system for Avr proteins. The ability of flax cells to take up secreted AvrM in the absence of flax rust indicates that a host-encoded uptake system for secreted Avr proteins exists. Full-length *AvrM* genes encoding wild-type versions of the secreted AvrM protein (i.e., containing the signal peptide) were expressed in flax leaves carrying the M resistance gene. The wild-type secreted protein (SP-WT) induced necrosis but a C-terminal ER retention signal (SP-HDEL) inhibited necrosis, while a nonfunctional signal (S-HDDL) did not. This indicates that the secreted AvrM protein was recognized after re-entering the plant via an unidentified host-encoded uptake system. Retention of the SP-HDEL in the inner membrane system prevented necrosis.  $\Delta$ SP-HDEL without the signal sequence was expressed in the cytoplasm without entry into and retention in the endomembrane system and induced necrosis (6).

by M, and importantly, the secreted form of the AvrM protein re-enters the host cytoplasm in the absence of the rust fungus. The implication of these data is that it is not necessary to postulate a specialized rust-encoded molecular machine for AvrM transport to the host cell during rust infection. The elucidation of the route of Avr protein is currently an important research target.

### THE MOLECULAR BASIS OF FLAX-FLAX RUST GENE-FOR-GENE SPECIFICITY AND AVR PROTEIN RECOGNITION

The most striking feature of flax-flax rust gene-for-gene interactions is the high degree of specificity of pathogen strain recognition determined by corresponding *R* and *Avr* genes. With cloned *R* and *Avr* genes, analysis of the determinants of specificity has become

possible. From the host perspective, sequence analysis of naturally occurring *R* genes and domain swap experiments between their alleles have identified two regions, the TIR and the LRR region that determine specificity, although it will be argued below that in terms of recognition functions, the LRR is the major domain (13, 25).

The role of the TIR region is probably in signaling and not pathogen recognition; however, variation in this domain can affect resistance gene specificity. For example, the L6 and L7 proteins differ by 11 polymorphisms exclusively in the TIR region and a chimeric *L2-L6* gene in which the region encoding the TIR domain of L6 is replaced by the same region of L2 encoding only 3 amino acid substitutions, also expresses L7 specificity. L6 and L7 are distinguished by rust strains that are either avirulent to both specificities or avirulent to L6 and virulent to L7 (Figure 2). However, when all 12 *AvrL567* variants were

transiently expressed in leaves of flax plants carrying *L6* or *L7*, the host reactions were qualitatively equivalent; all interactions that produced an HR with *L6* also did so with *L7* with one exception where the reaction of *L6* plants was very weak and undetected in *L7* plants (**Figure 5**). The reactions were quantitatively different, with *L6* always inducing the stronger, more rapid HR, indicating that *L6* and *L7* have the same recognition specificity and *L7* acts as a weaker allele. This is consistent with the weaker resistance of *L7* flax to avirulent rust infection; the incompatible reaction of *L7* is associated with an HR with some slight sporulation of the fungus, whereas the *L6* reaction is HR with no sporulation. So if the transient assay experiments indicate *L6* and *L7* have the same recognition specificity for cloned *AvrL567* variants, how are these alleles so clearly distinguished by rust infections? Genetic analysis of rust strains avirulent to *L6* but virulent to *L7* has shown that this pattern of reaction toward the R genes is not determined by the avirulence genotype (both rust genotypes carry functional *AvrL567* genes) but by a genetically unlinked inhibitor of avirulence gene (*I*). Rust strains that carry the *I* gene express *AvrL567* mRNA, so the *I* effect does not act at the level of transcription but may act to modify the Avr protein prior to secretion from the rust in such a way that it is not recognized by the *L7* protein. It is also possible that *I* protein is transferred to the host cell and inhibits recognition of *AvrL567* by *L7*. Because *L6* and *L7* alleles differ only in the TIR domain and this domain in animal systems is involved in signaling from receptors to downstream signal transducers, the *I* gene product may interfere with the postulated signaling process of the TIR domain and not Avr protein recognition by the host.

“Susceptibility” of a resistance gene to the inhibitor effect is not solely determined by the sequence of the TIR region. For instance, although resistance of the *L2-L6* chimera in **Figure 2** is inhibited by *I*, the *L2* gene itself is not. Also, although the *L10-AvrL10* inter-

action is also inhibited by *I*, the resistance encoded by a chimeric *L10-L2* gene carrying the TIR and most of the NBS domains of *L10* is not inhibited. The data suggest that “susceptibility” of an R protein to the *I* effect is controlled via an interaction between TIR and another region of the R protein and determined by sequence variation in both regions.

Three forms of the *I* gene have been identified in flax rust and these map to the same locus (17, 18, 23). Some rust strains carry a form that inhibits the action of five unlinked *Avr* genes, *AvrL567*, *AvrM1*, *AvrL1*, *AvrL8*, and *AvrL10* (and recognition by *L7*, *M1*, *L1*, *L8* and *L10* host genotypes), the second form inhibits only the action of *AvrM1* and the third “null” form inhibits none of these genes. The flax rust *I* gene is currently an important target for map-based cloning because the present data indicate that this function enables rusts to avoid detection by certain R proteins.

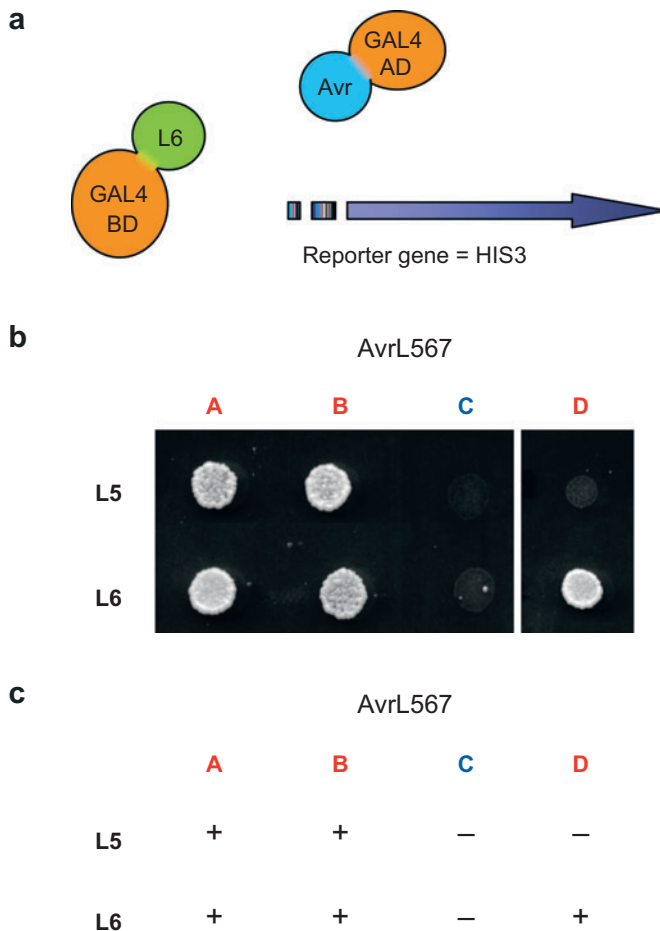
The major determinant of flax rust resistance protein specificity is the LRR domain, most likely due to its involvement in direct interaction with Avr proteins (6). Domain swap experiments that replaced the TIR and part of the NBS region of *L2* with the equivalent regions of *L6* and *L10*, while retaining the remainder of the NBS including the conserved R protein GLPL and MHD motifs, and LRR domains of *L2* expressed the *L2* specificity in transgenic plants (13). A more critical indication of the LRR role comes from comparison of the *L6* and *L11* alleles, which have identical promoters, encode identical TIR and NBS regions but LRR domains with 32 polymorphisms. These LRR differences account for the specific recognition of *AvrL567* by *L6* and *AvrL11* by *L11* (12). *AvrL567* and *AvrL11* are genetically unlinked and DNA probes from *AvrL567* do not hybridize to *AvrL11*. Although the *AvrL11* gene is not cloned yet, the DNA hybridization data indicate that *AvrL567* and *AvrL11* genes and their products are probably unrelated. So one or more of these 32 LRR domain polymorphisms would be involved in distinguishing the recognition of the unrelated Avr

proteins. Domain swap experiments between L6 and L11 indicate that the polymorphic residues that differentiate L11 from L6 are spread across the LRR region and that some polymorphisms necessary for L11 specificity are not necessary for L6 (12).

Further evidence for LRR specificity determination in flax rust resistance genes has been provided by a domain swap experiment that converted the *P2* specificity to *P* by exchange of only 6 polymorphic amino acids in the LRR domain (10). These polymorphic sites occur in the solvent exposed xxLxLxx (*x* = any amino acid, L = leucine)  $\beta$ -sheet- $\beta$  turn region of the individual LRR units. In this case, the corresponding avirulence genes recognized by *P* and *P2* are orthologous genes whose products differ by 36 amino acids. *P* recognizes AvrP but not AvrP123, whereas *P2* has the opposite specificity.

### DIRECT R-AVR PROTEIN INTERACTION UNDERLIES GENE-FOR-GENE SPECIFICITY IN FLAX

The proposed colocalization 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 (8). In these experiments the full-length *L5*, *L6* alleles and a chimeric construct *L6L11RV* (12), whose product differs from L6 by 11 amino acid differences derived from the 3 C-terminal LRR units of L11, and 12 *AvrL567* variants (*AvrL567-A* to *AvrL567-K*), were coexpressed in yeast two-hybrid assays (Figure 7). In these experiments there was close correspondence between the detection of a protein interaction in yeast and the induction of HR in planta, which 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 coexpression of L6 but not



**Figure 7**

Direct protein interaction underlies gene-for-gene specificity of flax resistance and flax rust avirulence genes. A larger data set is presented in (8). (a) Interactions between GAL4 activation domain (AD)-R protein fusions and GAL4 DNA binding domain (BD)-Avr protein fusions were assayed in yeast with the HIS3 reporter gene. Interactions allow growth of a histidine auxotrophic yeast strain on minimal medium lacking histidine. (b) Interactions between the *L5*, *L6*, and Avr protein variants *AvrL567-A*, -B, -C, and -D were indicated by growth of yeast on minimal medium lacking histidine. (c) Induction of necrosis on *L5* and *L6* flax genotypes. A complete correlation was observed between R-Avr interaction in yeast and induction of necrosis by expression of Avr variants in flax leaves carrying *L5* or *L6* resistance genes. (+ = necrosis, - = no necrosis).

*L5* with *AvrL567-D* induces HR in planta. Furthermore, the *L6L11RV* chimera interacts with only *AvrL567-J* in yeast and induces HR with only this Avr gene in planta. The observation that *L6L11RV* and *L6* differ only in the last three 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 the yeast two-hybrid 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, but the LRR domain is necessary for R-Avr interactions and that the minimum interacting deletions include both NBS and LRR domains (P.N. Dodds, unpublished).

These results are very similar to those reported by Ueda and colleagues (32) for the interaction between the TIR-NBS-LRR N protein of tobacco and the p50 fragment of the tobacco mosaic virus (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 and depended on the binding of ATP to the N protein and the minimum component of N for interaction was NBS-LRR. Gene-for-gene specificity was determined by demonstrating that replacement of proline with leucine at position 149 in p50, a mutation that occurs in the resistance breaking TMV strain Ob, abolished the interaction with N.

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 (P.N. Dodds, unpublished). However, whereas M is approximately 80% identical to L5 and L6, AvrL567 and AvrM proteins are unrelated (6). 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 flax R proteins directly interact with their corresponding Avr proteins, the picture is that NBS-LRR proteins can interact with diverse ligands and that the LRR region is highly flexible in an evolutionary sense with its capacity to recognize by direct interaction diverse pathogen ligands when coupled with the NBS domain. The L5 and L6 proteins, which interact with an overlapping set of *AvrL567* proteins, are among the most sequence-diverged L proteins with 87 polymorphisms, 59 in the LRR. This suggests that the common interactions that these proteins have with Avr proteins may be controlled by shared sequences in the LRR region (and possibly NBS?) or that the two proteins recognize similar ligands as a result of convergent evolution.

## RESISTANCE AND AVIRULENCE GENE EVOLUTION

In most of the comparisons between *L* alleles and between genes at the *N* and *P* loci, sequence variation is spread across the coding regions of the alleles with the highest levels of DNA sequence diversity in the LRR encoding domains, consistent with its role in specificity determination (9–11, 13). Analysis of the base substitutions indicated that the genes have undergone selection to encode amino acid diversity in this region, particularly in the solvent-exposed xxLxLxx motif of the LRR repeats, which is also consistent with the involvement of this domain in controlling specificity through Avr protein interactions. One part of the TIR coding sequence of the *L* alleles and also the CNL region of *P* locus genes showed evidence for diversifying selection, whereas the NBS encoding domain was subjected to purifying selection, consistent with a conserved enzymatic function of the latter (25). For the TIR region of the *L* alleles, there is indirect evidence from recombinant alleles that the TIR domain interacts with other regions of the *L* protein and that

this interaction is necessary for function. For example, the *suL10* (*suppressed L10*) allele, derived from crossing over between *L2* and *L10* alleles and that encodes a protein with the TIR of *L2* and NBS-LRR domains of *L10*, has no detected resistance function. *L10* and *suL10* differ by 5 residues in the TIR region derived from *L2*, and analysis of the differences in the DNA sequences encoding these polymorphic sites in *L* alleles indicates diversifying selection has acted in their evolution (25). These polymorphic TIR region residues could be involved in direct interactions (together with the LRR) with Avr proteins, although yeast two-hybrid results indicate that the TIR is not required for *L6*-Avr*L567* interactions. The TIR may also physically interact with other regions of the R protein (intramolecular interactions). If these interactions involved, for example, polymorphic LRR residues, selection for diversity in the LRR region to engage different Avr ligands could then select for appropriately coadapted sequences in the TIR region that stabilized the intramolecular interactions, thus accounting for the signature of diversifying selection in the TIR region. At this time, we have not been able to detect these postulated interactions using yeast two-hybrid assays (P.N. Dodds, unpublished).

Significantly, the sequences of the corresponding Avr*L567* genes are also highly variable with 35 polymorphic amino acid sites in the 127-residue protein with strong indications in the DNA sequences for diversifying selection. The evolutionary history of the R genes and their corresponding Avr genes is consistent with a coevolutionary arms race between the host and its obligate rust pathogen. A plausible evolutionary scenario is that effector proteins that enhance virulence evolved in flax rust, that receptor R proteins evolved in flax, and that the virulence effectors subsequently evolved to avoid detection but retain an as-yet unknown virulence function that is important for pathogen fitness. This view is supported by the observation that virulence forms of Avr*L567* locus are expressed and encode full-length protein variants and are not

associated with null alleles (e.g. deletions, internal frame shifts).

One aspect of the Avr*L567* locus that appears difficult to reconcile with this model is that all avirulence haplotypes carry two or more genes that are each recognized in transient expression assays in flax. For example, the rust strain Fi carries Avr*L567*-*A*, -*B*, -*F*, and -*L* that are each recognized by *L6* (8). So, to escape recognition by *L6* while maintaining the postulated virulence functions, simultaneous changes in all four genes are required. Each of the three examined virulence haplotypes (Figure 4) carry one or two virulence variants. These virulence haplotypes could arise by meiotic recombination during the flax rust sexual cycle that separates virulence forms, which arise by mutation, from avirulence forms, leading to virulence haplotypes that escape host R gene detection. At the population level, the different avirulence/virulence haplotypes are probably maintained by balancing selection with a host population that is also highly polymorphic for resistance/susceptibility.

The avirulence haplotype complexity also raises the question of the selective value of multiple avirulence variants at each haplotype and whether the postulated virulence function of each variant is identical or different. For instance, if the function of these effector proteins is to alter the expression of different host genes for the pathogen's advantage—for example, modulating expression of defense genes or metabolic genes supplying nutrients to the rust—then selection towards interaction with different host targets could account for the observed diversified genes and multiple gene haplotypes.

## THE EVOLUTION OF RESISTANCE GENE SPECIFICITIES FOR AGRICULTURE

The holy grail in the resistance gene field is the experimental generation of new resistance specificities, particularly for transgenic

application in crop protection. However, the term “specificity” can have several interpretations depending on the test applied. At the simplest level, two genes or alleles have different resistance specificities if these genes alone allow host genotypes to be distinguished on the basis of resistance/susceptibility phenotypes after inoculation with two or more pathogen isolates. On this basis, *L6* and *L7* can be distinguished. However, using a more sophisticated test based on coexpression of cloned *L6* or *L7* and corresponding Avr genes in transient assays, the specificities cannot be clearly distinguished and the differentiation by rust testing is, as described earlier, determined by the rust *I* gene whose effect is associated with sequence differences in the proposed signaling TIR domain. In a second example, *L6* and the chimeric resistance allele *L6L11RV* can also be distinguished by the simple rust inoculation tests. For example, *L6* confers resistance to rust strains CH5 and Bs1, whereas *L6L11RV* confers resistance to only Bs-1 (12). Although the two alleles can be clearly distinguished and no other naturally occurring allele confers the same pattern of rust strain recognition, there has been debate as to whether the recombinant *L6L11RV* constitutes a “new specificity.” Closer analysis by transient expression of *L6* and *L6L11RV* together with the seven *AvrL567* avirulence gene variants indicated that whereas *L6* recognized all, *L6L11RV* only recognized *AvrL567-7*, one of two Avr variants in the strain Bs-1 that is avirulent to the chimeric allele (8). Therefore, the exchange of the last 11 polymorphisms of *L6* with those of *L11* has altered the specificity of *L6* by reducing the range of *AvrL567* variants recognized by the chimeric protein. Purists in this area often express the following requirement for a new specificity derived by recombination between alleles: A new specificity must confer resistance to pathogen strains that are virulent to the input alleles. The *L6*, *L6L11RV* example does not fit this definition. Strictly speaking, even this definition is potentially inadequate if specificity is taken to reflect receptor-ligand

recognition. For example, a recombinant between *L7* and *L11* could produce the *L6* sequence ( $L11 = L6$  in the TIR+NBS,  $L7 = L6$  in the NBS+LRR). Available rust strains carrying *AvrL567*, *avrL11*, and *I* are virulent to *L7* and *L11* but avirulent to *L6* so by this definition, such a recombinant would be “new.” However the coexpression assay of the parental specificities with *AvrL567* variants would not distinguish *L7* and *L6* (or any other parental and derivative alleles where inhibitor genes are active). The best way to distinguish a newly generated specificity is by using coexpression of the input and recombinant alleles and the cloned *Avr* genes. Regardless of definitions, in a practical sense, a new specificity is of use when it provides resistance to a pathogen for which no effective R gene is known. Recently, such a new specificity has been generated by random mutation in the LRR region of the Rx protein for resistance to potato virus X (14). The new specificity provides resistance to “standard” virus isolates and also to Rx resistance-breaking strains of the virus, thus providing the first example of developing synthetic resistance genes by modifying a cloned R gene. Further efforts to evolve new R gene specificities in vitro will be facilitated by yeast two-hybrid selection for interactions between mutant R genes and a targeted pathogen effector molecule.

Sequence comparisons of cloned R genes have provided insight into the evolution of naturally occurring resistance specificities. Point mutations have produced multiple polymorphic sites in the gene sequence and these have been shuffled by crossing over or gene conversion events giving rise to a series of variants with patchwork regions of diversity shared by different alleles/paralogs. Single intra-allelic crossing-over has been observed experimentally at frequencies of about 1 per 1000 gametes; however, many of these recombinants and those derived from in vitro exchanges have no detectable function, probably because intramolecular associations necessary for function or stability are disrupted (25). If evolution of resistance genes involved

sequential cross-overs, nonfunctional variants would arise as intermediates in evolution. Gene conversions that exchange small regions of the LRR without causing disruptions in function would likely provide a more direct route to new specificities. At complex loci such as *N* in flax, there is evidence for past exchanges of sequence between paralogs but the rate of exchange is highly dependent on high levels of sequence relatedness of the paralogs (9). These loci can maintain a reservoir of diversity that can be shuffled by recombination. However, it is also clear that when point mutation increases the sequence differences between paralogues, sequence exchanges are very infrequent and genes in the haplotype become isolated (9, 21).

One additional striking feature of the *L* and *M* LRR coding regions (but not the *N* and *P* genes) is their extreme evolutionary plasticity in terms of size. The LRR of *L*, *L3*, *L4*, *L5*, *L6*, *L7*, *L9*, *L10*, and *L11* consist of 26 imprecise direct repeats. Within this region occur two larger-order direct repeats of 450 bp (encoding 6 LRR units) of 80% sequence identity that have arisen by a duplication event. *L2* has 4 of these repeats, whereas *L1* and *L8* each have only one, probably resulting from interrepeat recombination (*L1* and *L2*) or deletion in *L8*. Such an event has been observed in “real time”; a spontaneous loss-of-function allele of *M* involved loss of a repeat through unequal crossing over (2). LRR proteins commonly fold into a horseshoe-like curve, and the changes in spacing between critical binding residues produced by expansion/reduction of LRR regions may well assist in optimization of binding to ligands with a range of sequences and sizes.

## DIRECT AND INDIRECT RECOGNITION OF PATHOGENS BY R PROTEINS

Either direct or indirect interactions occur between different resistance proteins and their corresponding avirulence proteins with the flax-flax rust L567/AvrL567 system being an

example of the former (8). Indirect interactions, so far most extensively documented in plant-bacterial interactions, involve resistance proteins that detect modifications of other host proteins induced by pathogen avirulence (effector) proteins. For example, in *Arabidopsis-Pseudomonas* systems, the recognition of avirulence proteins avrRpt2 and AvrRpm1 by corresponding resistance proteins RPS2 and RPM1 is indirect, via R protein monitoring of the Avr-induced changes to the host protein RIN4 (3, 26, 27). In these cases R proteins detect the biochemical activity of Avr proteins. A potential selective penalty on pathogens recognized indirectly is that escaping recognition requires loss of effector function and probable loss of pathogenic fitness. Such fitness reduction is likely to be more serious for obligate biotrophs with no saprophytic phase than non-obligate pathogens. Consequently, we predict that direct R-Avr protein interactions will be more common in obligate biotrophic interactions and that effector-induced modification of host target proteins may be very subtle (for example, inhibition of the active site of a host enzyme) and difficult for host plants to detect by indirect means. In these cases, selection on the host for direct recognition of pathogen effectors and subsequent selection on pathogen effectors for changes that cause loss of recognition but not effector function is envisaged.

Based on the *Arabidopsis-Pseudomonas* systems and the flax-flax rust system, predictors of indirect R-Avr interactions are likely to be low levels of polymorphism in R and corresponding Avr genes in host and pathogen populations, respectively. For example, RPM1 and AvrRPM1 are characterized by presence-absence polymorphisms. Based on the flax rust system, predictors of direct interactions are diversifying selection in both host resistance genes and corresponding pathogen *Avr* genes, which suggest situations where pathogen effectors are selected for loss of recognition and retention of function, and host resistance genes are selected for ability to recognize evolving effector diversity. These predictions

can be tested in several interactions currently being investigated, for example *RPP13/Atr13* (*Arabidopsis/Hyaloperonospora*), *RPP1/ATR<sup>NdW<sub>SB</sub></sup>* (*Arabidopsis/Hyaloperonospora*) and *Mla/Avr-a10-k1* (barley/*Blumeria*) where the criterion of R gene and Avr gene diversity has been met but the nature of the recognition (direct or indirect) has not yet been reported. (1, 29, 30).

## PERSPECTIVES

The work in the flax rust system and other biotrophic host-pathogen interactions has raised a number of critical questions for future investigation. How general are and what function do *Inhibitor of avirulence* proteins have in plant pathogen interactions? Rehmany et al. (29) reported a potential *I* gene effect in the *Arabidopsis-Hyaloperonospora* interaction. Although the avirulence gene *ATRI<sup>NdW<sub>SB</sub></sup>* from the Emco5 pathogen strain is recognized by *RPS1-W<sub>SB</sub>* in transient assays, the Emco5 strain is virulent to Ws-0 plants, the source of the *RPS1-W<sub>SB</sub>* gene. Genetic analysis of Emco5 is required to confirm the presence of an *Inhibitor of avirulence* gene.

Do fungal and oomycete Avr proteins have a role in virulence (effector activity) and what are these functions and host targets? How do these effectors enter plant cells? Signals for uptake of secreted proteins by host cells have been identified in oomycete effectors (29) but

not in fungal Avr proteins. Among the proteins secreted by the haustoria of the rust fungus *Uromyces fabae*, some but not all enter the host cell, which demonstrates the existence of an uptake mechanism capable of discriminating between these secreted proteins (20). Recently derived genome sequences of two *Phytophthora* species encode about 350 secreted proteins in each species carrying the host uptake signal that are likely to act inside the host cell (31). The genome sequence of the maize pathogenic fungus *Ustilago maydis* also identified a large number of secreted proteins, some of which have demonstrated virulence functions. Even with the currently small sample of sequenced plant pathogen genomes, two remarkable features of the eukaryotic plant pathogens proteome expressed and secreted during host infection are the large numbers of proteins with no clear relationship to previously characterized proteins (19, 31) and the large number of sequences that are specific to individual species of the same genus (31). The question is whether the diversity represents adaptation of the pathogen towards distinct host species. If so, this may reflect differences in virulence targets or convergent evolution of different effectors towards the same host targets. The genome sequences of closely related species in the same genus that infect the same host species will be of great interest. The three *Puccinia* species that infect wheat fit this bill.

## DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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## Errata

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