

# Haustorially Expressed Secreted Proteins from Flax Rust Are Highly Enriched for Avrulence Elicitors<sup>W</sup>

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Rust fungi, obligate biotrophs that cause disease and yield losses in crops such as cereals and soybean (*Glycine max*), obtain nutrients from the host through haustoria, which are specialized structures that develop within host cells. Resistance of flax (*Linum usitatissimum*) to flax rust (*Melampsora lini*) involves the induction of a hypersensitive cell death response at haustoria formation sites, governed by gene-for-gene recognition between host resistance and pathogen avirulence genes. We identified genes encoding haustorially expressed secreted proteins (HESPs) by screening a flax rust haustorium-specific cDNA library. Among 429 unigenes, 21 HESPs were identified, one corresponding to the *AvrL567* gene. Three other HESPs cosegregated with the independent *AvrM*, *AvrP4*, and *AvrP123* loci. Expression of these genes in flax induced resistance gene-mediated cell death with the appropriate specificity, confirming their avirulence activity. *AvrP4* and *AvrP123* are Cys-rich proteins, and *AvrP123* contains a Kazal Ser protease inhibitor signature, whereas *AvrM* contains no Cys residues. *AvrP4* and *AvrM* induce cell death when expressed intracellularly, suggesting their translocation into plant cells during infection. However, secreted *AvrM* and *AvrP4* also induce necrotic responses, with secreted *AvrP4* more active than intracellular *AvrP4*, possibly as a result of enhanced formation of endoplasmic reticulum-dependent disulfide bonds. Addition of an endoplasmic reticulum retention signal inhibited *AvrM*-induced necrosis, suggesting that both *AvrM* and *AvrP4* can re-enter the plant cell after secretion in the absence of the pathogen.

## INTRODUCTION

Rust fungi (order Uredinales) use one of the most specialized infection strategies of plant pathogens. These obligate biotrophs obtain their nutritional resources from the living cells of their host and are completely dependent on living plant tissue for their growth and propagation. Fungal hyphae grow in the intercellular space of the leaf but form a close association with host plant cells through haustoria. These specialized infection structures differentiate from haustorial mother cells and penetrate the plant cell wall. The plant cell membrane is invaginated and forms an extrahaustorial membrane, with a region between this and the fungal membrane termed the extrahaustorial matrix. Haustoria are thought to play the primary role in nutrient acquisition from the host (Hahn and Mendgen, 2001) and apparently also influence host metabolism and defense responses (Voegelé and Mendgen, 2003) and induce the reorganization of the host cell cytoskeleton and nuclear DNA (Kobayashi et al., 1994; Heath, 1997). Thus, the haustorium–host cell interface appears to be a dynamic interaction involving extensive molecular trafficking and signaling processes. However, the nature of these ex-

changes remains unknown. Other obligate biotrophs, such as the oomycete downy mildew pathogens and the ascomycete powdery mildews, have independently evolved a similar infection process (Panstruga, 2003), although the powdery mildews propagate aerial hyphae on the leaf surface and form haustoria only in epidermal cells. Some hemibiotrophic fungi and oomycetes, such as *Phytophthora*, also form haustoria during their initial stage of infection, but they later enter a necrotrophic stage during which they induce host cell death (Perfect and Green, 2001).

Frequently, plant disease resistance relies on the recognition of pathogen avirulence (Avr) products by host Resistance (R) proteins, which induces a hypersensitive response (HR). Avr proteins are generally thought to be pathogenicity effectors with positive roles in establishing infection, which the plants' R protein surveillance mechanism has evolved to recognize as indicators of invasion. The nature and functions of these effector molecules differ between pathogen classes. For instance, most bacterial Avr proteins are delivered directly into the plant cytoplasm via the type III secretion system (Lahaye and Bonas, 2001). Although the functions of many of these effectors remain unknown, some are targeted to the host nucleus and may act as transcription factors (Lahaye and Bonas, 2001; Deslandes et al., 2003), whereas others are proteases that cleave specific cytoplasmic host proteins (Orth et al., 2000; Axtell et al., 2003; Shao et al., 2003; Coaker et al., 2005). These bacterial Avr proteins are generally recognized by intracellular R proteins containing nucleotide binding site (NBS) and leucine-rich repeat (LRR) domains (Luderer and Joosten, 2001). By contrast, Avr proteins from extracellular fungal pathogens are secreted into the apoplast. These fungal

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effectors, such as AVR2 (Luderer et al., 2002), AVR4 (Joosten et al., 1994), and AVR9 (van den Ackerveken et al., 1992) from *Cladosporium fulvum*, NIP1 from *Rhynchosporium secalis* (Rohe et al., 1995), and SIX1 from *Fusarium oxysporum* f sp *lycopersici* (Rep et al., 2004), are Cys-rich, and for most of these proteins the formation of disulfide bonds is critical for their structure and stability in the protease-rich apoplast (van den Hoooven et al., 2001; van den Burg et al., 2003; van't Slot et al., 2003). The biochemical activities of some of these proteins are known. For instance, AVR4 is a chitin binding protein that protects fungal cell walls from degradation by plant chitinases (van den Burg et al., 2003), and AVR2 is an inhibitor of the extracellular Cys protease Rcr3 in tomato (*Lycopersicon esculentum*) (Rooney et al., 2005). R proteins recognizing the *Cladosporium* extracellular Avr products are membrane-bound receptors with extracellular LRR domains (Luderer and Joosten, 2001).

In contrast with these well-studied pathogen classes, there is a dearth of information concerning virulence and avirulence factors and pathogenicity mechanisms of rust and mildew pathogens, largely because of the difficulties of studying these obligate biotrophs. Identification of the first rust Avr proteins, the AvrL567 proteins of the flax rust *Melampsora lini* (Dodds et al., 2004), led to the realization that these biotrophic fungi, like bacterial pathogens, may deliver proteins directly into the plant cytoplasm. AvrL567 proteins are expressed in haustoria and contain predicted signal peptides, suggesting that they are secreted into the extrahaustorial matrix. However, expression of these proteins in the plant cytoplasm induces an HR dependent on the cytoplasmic NBS-LRR L5, L6, or L7 resistance proteins of flax (*Linum usitatissimum*). This finding suggests that there may be a specific translocation mechanism that delivers these proteins across the plant-derived extrahaustorial membrane and into the host cell cytoplasm during rust infection. However, the nature of this mechanism remains unresolved. Avr proteins recently identified from biotrophic and hemibiotrophic oomycetes, such as ATR13 and ATR1<sup>NdWsB</sup> from *Hyaloperonospora parasitica* (Allen et al., 2004; Rehmany et al., 2005), Avr1b from *Phytophthora sojae* (Shan et al., 2004), and Avr3a from *Phytophthora infestans* (Armstrong et al., 2005), also contain signal peptides. Like AvrL567, ATR13, ATR1<sup>NdWsB</sup>, and Avr3a trigger a HR response when localized inside the host cell, suggesting that they may be translocated during infection. A conserved motif in these oomycete proteins is similar to a motif required for the transport of proteins secreted by the malaria parasite (*Plasmodium falciparum*) proteins across the erythrocyte vacuolar membrane (Hiller et al., 2004; Marti et al., 2004), suggesting the possibility of a conserved translocation mechanism used by these highly diverged organisms.

To further investigate rust fungal pathogenicity mechanisms, we have attempted to isolate additional Avr genes from flax rust. Because the HR that occurs during rust resistance appears to be focused on the emerging haustoria (Kobayashi et al., 1994; Heath, 1997), we reasoned that rust Avr proteins should be expressed in these structures. In addition, the precedent of AvrL567 and the observation that all 19 of the cloned flax *R* genes encode predicted cytoplasmic NBS-LRR proteins (Lawrence et al., 1995; Anderson et al., 1997; Dodds et al., 2001a, 2001b) suggested that these would be secreted proteins. Therefore, we generated a haustorium-specific cDNA library and searched for ESTs

encoding putative secreted proteins. Three of these cosegregated with Avr loci in an F2 mapping family, and transient expression assays showed that these genes function as avirulence determinants to induce *R* gene-dependent cell death in flax.

## RESULTS

### Isolation of Haustorially Expressed cDNAs Encoding Predicted Secreted Proteins

A haustorium-specific cDNA library was searched for ESTs encoding putative signal peptides. Haustorial cells were isolated from flax leaves infected with rust strain CH5 using affinity chromatography with a Sepharose-concanavalin A column (Hahn and Mendgen, 1992). The majority of isolated haustorial cells remained intact, and contaminating plant cells represented <1% of haustoria numbers. A cDNA library was constructed from haustoria RNA samples and consisted of  $3.5 \times 10^6$  independent clones. Preliminary sequencing revealed a high number of clones containing rRNA genes, and subsequent clones were pre-screened by colony blot hybridization using probes against a subset of flax rust rRNAs. A total of 822 clones were sequenced, of which 150 matched rRNA genes. The remaining 672 ESTs generated 429 unique sequences. BLAST (Altschul et al., 1997) searches against the public databases identified related sequences for 46% of the unique ESTs (E values  $< 1 \times 10^{-4}$ ). Approximately 5% of sequences were significantly similar to known plant genes and were probably derived from contaminating flax cells. Four ESTs were homologous with genes identified from a *Uromyces fabae* (broad bean rust) haustorium-specific library: *MAD1* (mannitol dehydrogenase; Voegelé et al., 2005); *BGL1* ( $\beta$ -glucosidase; Haerter and Voegelé, 2004); *HXT1* (hexose transporter; Voegelé et al., 2001); and *PIG28* (a putative cyclophilin; Hahn and Mendgen, 1997).

Among the initial sequences, three ESTs were found to encode the secreted flax rust AvrL567 proteins. To identify additional secreted proteins, we selected a subset of 167 unique ESTs based on the presence of a potential full-length open reading frame that included an initiating Met codon and lacked homology with proteins of known intracellular function (e.g., ribosomal proteins). To search for potential secretory signals, the predicted protein sequences of these cDNAs were analyzed with the SignalP 3.0 algorithm (Bendtsen et al., 2004; <http://www.cbs.dtu.dk/services/SignalP/>). This screen identified 20 sequences encoding proteins with predicted secretory signal peptides (Table 1). To support the SignalP results, the protein sequences for each of the haustorially expressed secreted proteins (HESPs) were also entered into other signal peptide-predicting software. iPSORT (Bannai et al., 2002) predicted 17 of 20 proteins to be secreted, and PSORTII (Nakai and Horton, 1999) predicted 19 of 20 with McGeoch's method. These HESPs encode small proteins (73 to 384 amino acids), most with no significant similarity to known protein sequences. Four HESPs were homologous with predicted fungal proteins of unknown function, and two contained motifs indicative of Kazal-type Ser protease inhibitors (C51 = AvrP123-A; Figure 1A) or trehalose phosphatases (C61). Eight HESPs encode Cys-rich proteins with even numbers of Cys residues that may be involved in disulfide bonds.

**Table 1.** Flax Rust HESPs

HESP No.	Redundancy <sup>a</sup>	Protein Size (Amino Acids)	No. of Cys Residues	Top Hit with BLASTP Search (Matches Are to Predicted Proteins)	E Value	Protein Signature <sup>b</sup>
178	1	146	8	<i>Magnaporthe grisea</i> (BAC65876)	1E-05	CFEM
270	1	267+				
327	1	79				
376	1	142				
379	1	205		<i>Ustilago maydis</i> (EAK82919)	3E-10	
417	1	126	4			
570	1	222				
642	1	140	6			
735	1	169				
767	1	85		<i>Cryptococcus neoformans</i> var <i>neoformans</i> (EAL23156)	4E-24	
897	1	142	10			
937	1	113				
C29	5	343				
C48	2	95	6			
C49	2	176	12			
C51	4	117	10			KAZAL
C55	3	122				
C61	5	327		<i>Yarrowia lipolytica</i> (CAG81011)	5E-13	Trehalose PPase
C63	3	197				
C66	3	73	8			

<sup>a</sup> Number of clones in 822 sequences from a haustorium-specific cDNA library.

<sup>b</sup> Protein signatures are as follows: CFEM, fungal-specific eight-Cys-containing domain of extracellular membrane proteins, found in some proteins with proposed roles in fungal pathogenesis; KAZAL, family of Ser protease inhibitors; Trehalose PPase, trehalose phosphatases catalyze the dephosphorylation of trehalose-6-phosphate to trehalose and orthophosphate.

### The *AvrM*, *AvrP4*, and *AvrP123* Loci Encode HESPs

DNA gel blot hybridization confirmed that all of the HESPs were derived from the rust genome. Sixteen HESPs detected restriction fragment length polymorphisms that were mapped among a flax rust F2 family of 74 individuals (self-progeny of rust CH5) that segregate for 16 different avirulence specificities located at 10 loci (Lawrence et al., 1981a). Two HESPs, C29 and C48, detected restriction fragment length polymorphisms that cosegregated with *Avr* genes corresponding to the flax resistance genes *M* and *P4*, respectively. A third HESP, C51, detected several restriction fragment length polymorphisms that either cosegregated with or were closely linked to a complex *Avr* locus corresponding to the *P1*, *P2*, and *P3* resistance genes in flax. These avirulence specificities are tightly linked but have been separated by recombination or mutation events in several individuals found in a large screening procedure (Lawrence et al., 1981b). The remaining 13 HESPs showed no genetic association with other known *Avr* loci segregating in this rust family. The HR is one of the primary manifestations of R–Avr recognition, and in planta expression of corresponding *R* and *Avr* genes has been shown to induce a HR-like cell death (Erickson et al., 1999; van der Hoorn et al., 2000; Allen et al., 2004; Dodds et al., 2004). Therefore, we generated T-DNA constructs in which the *Avr* gene candidates were under the control of the 35S promoter and used *Agrobacterium tumefaciens* infiltration to express these constructs in plants containing the corresponding resistance genes. Expression of C51 induced a HR-like necrotic response on near-isogenic

flax plants containing the *P1* and *P2* resistance genes but not on those containing *P3* or the recurrent backcross parent, Bison (Figure 1B). This finding indicates that this gene is indeed an active member of the *AvrP1*, *P2*, *P3* complex locus, and we have designated it *AvrP123-A*. Hybridization of this probe to genomic DNA gel blots indicated that several additional homologs occur at this locus, but these have not been examined further in this study. In planta expression assays (see below) also showed that the C29 and C48 genes triggered *R* gene-dependent cell death with the appropriate specificity; therefore, we designated them *AvrM* and *AvrP4*. These two *Avr* loci are described in greater detail below.

### The *AvrM* Locus Contains Multiple Homologs

DNA gel blot analysis detected up to eight DNA fragments hybridizing to the *AvrM* probe in rust strain CH5, depending on the restriction enzyme used. Most of the fragments segregated with the avirulence allele, whereas a single fragment occurred at the virulence allele (data not shown). Sequence analysis of RT-PCR products amplified from RNA of leaves infected with an F2 rust strain homozygous for the virulence allele detected a single sequence variant, *avrM*. By contrast, four sequence variants (*AvrM-A*, *-B*, *-C*, and *-D*) were detected in RT-PCR products amplified from leaves infected with a homozygous avirulent F2 rust strain. Screening the haustorium cDNA library with the C29 probe identified one additional full-length sequence variant



**Figure 1.** The Kazal-Like *AvrP123-A* Is Recognized by *P1* and *P2* Resistance Genes in Flax.

**(A)** Amino acid sequence of the predicted *AvrP123* protein. The predicted signal peptide is underlined, and the consensus residues of the KAZAL family of Ser protease inhibitors are indicated by black arrowheads with the residue at the P1 active site indicated. The Cys residues and the GIAR motif identified by Multiple Expectation Maximization for Motif Elicitation (MEME) analysis are shaded in gray.

**(B)** Leaves of four near-isogenic flax lines containing *P1* (B10×P1), *P2* (B4×P2), *P3* (B13×P3), or no *P* resistance gene (Bison) were infiltrated with *A. tumefaciens* cultures containing T-DNA plasmids encoding the full-length *AvrP123-A*. The photograph was taken 10 d after infiltration.

(*AvrM-E*) as well as two partial cDNA clones with further sequence differences that may represent additional *AvrM* variants. Thus, the *AvrM* avirulence allele contains at least five closely related sequences, whereas a single copy occurs at the virulence allele. The *AvrM* proteins show no significant sequence similarity (BLASTP; Altschul et al., 1997) to other known proteins or peptide sequence motifs.

The six predicted *AvrM* proteins contain several sequence differences and also vary in size (Figure 2). *AvrM-B* and *-C* both contain 377 amino acid residues, whereas *AvrM-A* has a 34-amino acid C-terminal truncation, resulting in a 343-residue protein. *AvrM-D* shares this truncation but also contains a 7-amino acid insertion (corresponding to a 21-bp perfect repeat in the coding sequence), resulting in a 350-amino acid protein. *AvrM-E* also contains this insertion but is truncated to 212 amino acids as a result of a nucleotide change that introduces a premature stop codon in the coding sequence. The *avrM* protein derived from the virulent rust parent contains 314 amino acid residues as a result of two in-frame deletions of 207 and 3 bp. In addition to these large structural differences, there are 14 polymorphic sites within the protein sequence. *AvrM-B* and *-C* share the greatest sequence similarity, with only 3 amino acid differences between them; the greatest variation is seen with *avrM*, which has two deletions and 10 unique amino acid differences (Figures 2A and 2C). The first 28 residues of all of these proteins are not polymor-

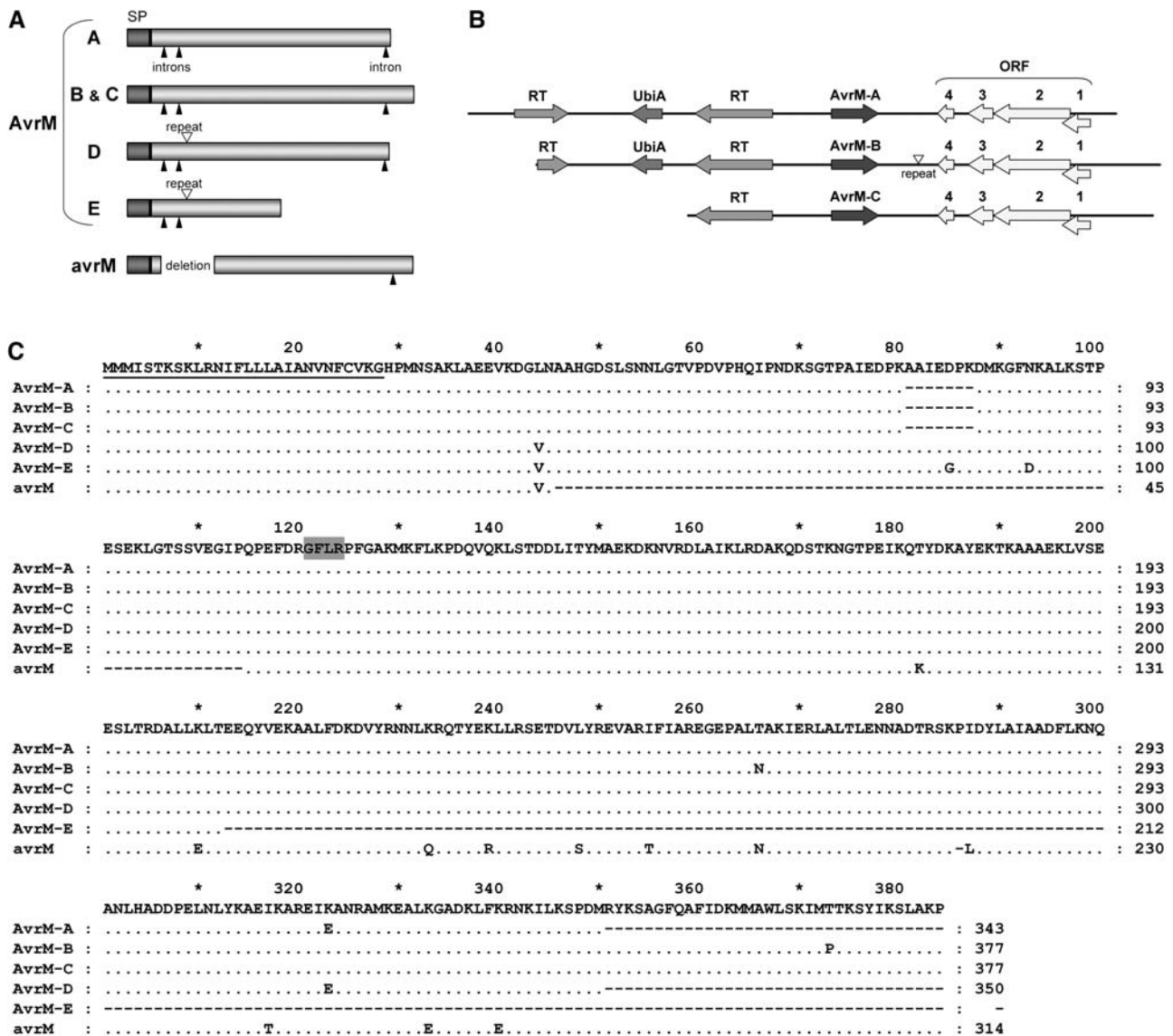
phic and are predicted to be a cleaved secretion signal peptide. The presence of upstream stop codons in the cDNA sequences indicates that the predicted open reading frames are full length.

An *AvrM* probe was used to isolate clones from a genomic DNA library prepared from the rust strain CH5. Of five sequenced clones, three contained *AvrM-C* (the largest sequenced region being 11.8 kb) and the remaining two encoded *AvrM-A* (16.3 kb sequenced) and *AvrM-B* (15.9 kb sequenced). Because these sequences do not overlap, the *AvrM-A*, *-B*, and *-C* genes must be separated by at least 10 to 15 kb on the chromosome. Three introns of 74, 70, and 98 bp are present within the coding region, although the first two intron sites are absent from *avrM* as a result of the large internal deletion within the genomic DNA (Figure 2A). Significant sequence identity (99.9%) in the DNA surrounding the *AvrM* genes suggests that they evolved from large duplication events (Figure 2B). Upstream of the *Avr* open reading frame are two regions with similarity to reverse transcriptase sequences and a predicted gene related to UbiA prenyltransferase genes. Downstream of the *Avr* genes are four codirectional open reading frames that may represent a single open reading frame separated by introns. No proteins with significant similarity to these sequences were detected in public databases.

### Transient Expression of *AvrM* Genes in Flax Causes *M* Gene-Dependent Cell Death

We used *A. tumefaciens* infiltration to transiently express the *AvrM* genes in flax plants with or without the *M* resistance gene. Two sets of T-DNA plasmids were constructed with cDNA clones of each of the *AvrM* genes, one containing the entire open reading frame and another that excluded the predicted signal peptide but included an additional ATG start codon. All constructs contained the 35S promoter of *Cauliflower mosaic virus* to drive transcription and the nopaline synthase terminator of *A. tumefaciens*. *A. tumefaciens* strains containing *AvrM* constructs were infiltrated into the leaves of four flax genotypes: Bison (m/m) and its near-isogenic line B12×M containing the *M* gene, Hoshangabad (m/m), and a transgenic derivative transformed with the *M* gene (Hosh::M) (Anderson et al., 1997) (Figure 3). Transient expression of *AvrM* genes without the predicted signal peptides in the leaves of Bison and Hoshangabad gave no phenotype. However, expression of *AvrM-A*, *-B*, *-C*, or *-D* in leaves containing the *M* gene, either B12×M or Hosh::M, induced a necrotic response indicating that all four proteins are recognized by the *M* protein. Expression of *avrM* encoded by the homologous gene from the virulent parental rust strain did not induce necrosis in any flax genotype. Similarly, the truncated *AvrM-E* protein was not recognized by *M*. RNA gel blot analysis showed that the *AvrM* constructs were each expressed at similar levels in *A. tumefaciens*-infiltrated leaves, except for *avrM*, which showed a reduced level of expression (data not shown).

The necrotic response seen in the infiltration experiments varied between the *AvrM* genes and between the different *M* gene flax lines (Figure 3). In B12×M, *AvrM-A* gave the quickest necrotic response, followed by *AvrM-D*, whereas the necrosis seen with *AvrM-B* and *-C* was considerably slower to develop (8 to 10 d compared with 3 to 4 d). When these genes were transiently expressed in Hosh::M, the overall response was reduced and



**Figure 2.** Characterization of the Flax Rust *AvrM* Locus.

**(A)** Scheme showing the variation at the *AvrM* locus. For each open reading frame, the signal peptide (SP) and intron positions in the corresponding genomic DNA (black arrowheads) are shown. The positions of the 207-bp deletion in *avrM* and the 22-bp repeat (white arrowhead) in *AvrM-D* and *-E* are indicated.

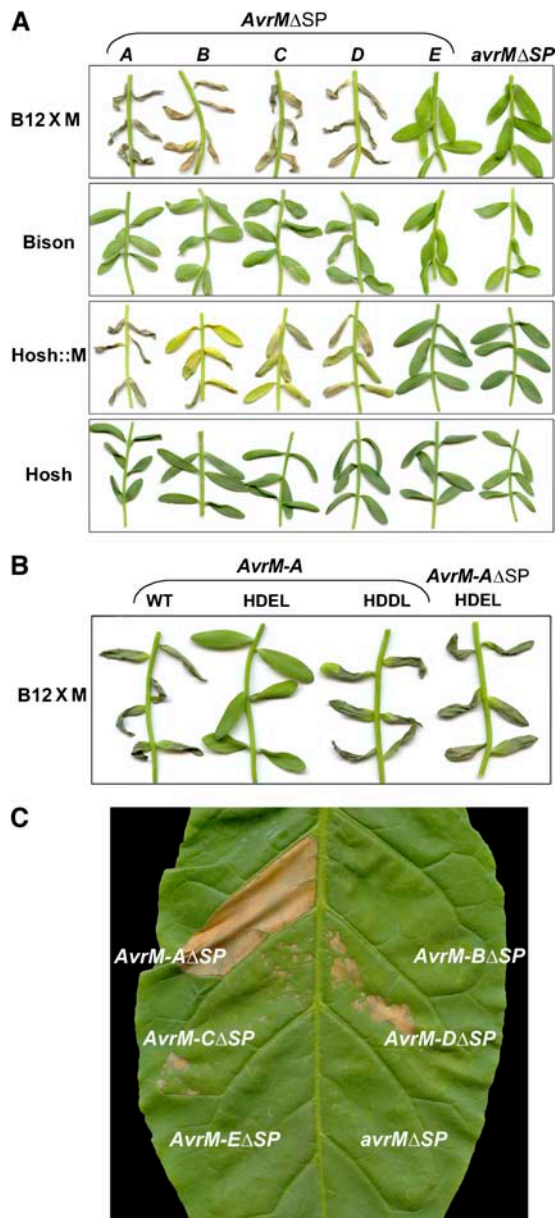
**(B)** Scheme of three avirulence genes at the *AvrM* locus. Three regions from the avirulence allele (16.3, 15.9, and 11.8 kb) contain the *AvrM-A*, *AvrM-B*, and *AvrM-C* genes, respectively. The positions of several common features surrounding the *Avr* open reading frames are shown. Upstream are two regions homologous with a reverse transcriptase (RT) and a third region related to UbiA prenyltransferase (UbiA). Downstream are four codirectional open reading frames (ORF; no similarity to database sequences) that may represent a single open reading frame separated by introns. The position of a 22-bp repeat that occurs downstream of *AvrM-B* is shown as a white arrowhead.

**(C)** Amino acid sequence alignment of the predicted *AvrM* proteins. Only those amino acids that differ from the consensus (top line) are shown, with identical residues indicated by dots and gaps indicated by dashes. The signal peptide is underlined, and the GFLR motif identified by MEME analysis is shaded gray.

delayed by 3 to 4 d, except for that of *AvrM-A*, which gave an equivalent and strong response in both flax lines. Induction of *R* gene-dependent necrosis by *AvrM* lacking the predicted signal peptides suggests that recognition occurs within the plant cell, which agrees with the predicted cytoplasmic location of the M

resistance protein. However, expression of the full-length *AvrM* genes including the signal peptides also induced a necrotic response. In the case of *AvrM-A* and *-D*, this response was similar to that induced by the truncated genes, but for *AvrM-B* and *-C*, the response was slower to develop for the full-length





**Figure 3.** Transient Expression of *AvrM* Genes in Flax Causes *M* Gene-Dependent Necrosis.

**(A)** Leaves of four different flax genotypes with or without the *M* resistance gene were infiltrated with *A. tumefaciens* cultures containing T-DNA plasmids encoding *AvrM*-A, -B, -C, -D, -E, or *avrM*. Photographs were taken 12 d after infiltration. All constructs excluded the predicted signal peptide sequence but included an additional ATG start codon ( $\Delta$ SP). B12XM and Hosh::M are flax lines containing the *M* gene, whereas the Bison and Hoshgabad (Hosh) varieties do not.

**(B)** Leaves of flax variety B12XM, carrying the *M* resistance gene, were infiltrated with *A. tumefaciens* cultures containing T-DNA plasmids encoding the full-length wild-type *AvrM*-A protein, the full length *AvrM*-A with a C-terminal HDEL or HDDL extension, or the truncated *AvrM* $\Delta$ SP with the HDEL extension. The photograph was taken 4 d after infiltration.

**(C)** Leaves of transgenic tobacco W38 carrying the flax rust resistance gene *M* were infiltrated with *A. tumefaciens* cultures containing T-DNA

versions and was characterized by chlorosis rather than necrosis (see Supplemental Figure 1 online).

To investigate whether the leader peptide functions as a secretion signal in planta to direct the *AvrM* proteins through the endoplasmic reticulum (ER) secretory pathway, we expressed this protein with the HDEL tetrapeptide ER retrieval signal (Denecke et al., 1992, 1993) fused to the C terminus. If the full-length protein is secreted through this pathway, retention in the ER would prevent accumulation of the protein in the apoplast and subsequent reentry into the plant cytoplasm. Addition of the HDEL motif inhibited the necrotic response induced by the full-length *AvrM*-A protein (Figure 3B). However, the chemically similar tetrapeptide HDDL, which does not function as an ER retrieval signal in plants (Denecke et al., 1992, 1993), did not affect *AvrM* recognition. Furthermore, recognition of the nonsecreted version of *AvrM*-A was not affected by the addition of HDEL (Figure 3B). This finding demonstrates that the presence of this motif does not interfere with *AvrM* recognition, and the inhibition of HR induction by the full length *AvrM*-A-HDEL protein is likely attributable to the retention of the protein in the ER. These results confirm that the predicted signal peptide is functional in plants and suggest that secreted *AvrM* protein reenters the plant cell from the apoplast.

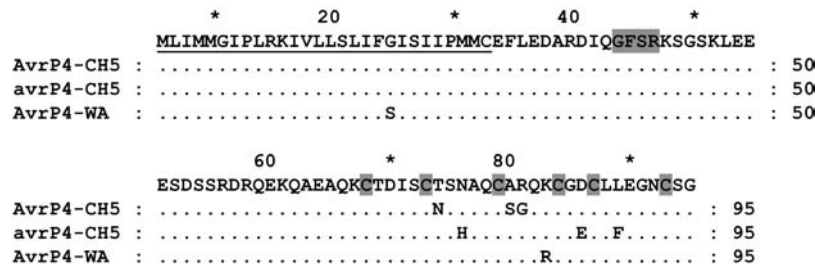
The flax rust *AvrL567* genes were found to induce a HR when coexpressed in tobacco (*Nicotiana tabacum*) with the flax resistance gene *L6* (Dodds et al., 2004). To determine whether *AvrM* could also give a recognition interaction in an unrelated species, they were transiently expressed in transgenic W38 tobacco containing the *M* gene (W38::M). Expression of *AvrM*-A gave a very strong necrotic response, *AvrM*-C and -D gave much weaker responses, and no necrotic response was seen for *AvrM*-E and *avrM* (Figure 3C). Although no necrosis was seen for *AvrM*-B, these results generally reflected the recognition specificity seen in flax. No response was seen with any of the infiltrated *AvrM* genes in nontransgenic W38 tobacco (data not shown).

#### ***AvrP4* Encodes a Small Cys-Rich Secreted Protein**

In contrast with the complex *AvrM* locus, DNA gel blot analysis with HESP C48 detected a single hybridizing fragment segregating with each allele of *AvrP4*. RT-PCR amplification of RNA from leaves infected with F2 rust strains homozygous for the avirulence (*AvrP4*) or virulence (*avrP4*) alleles identified a single sequence variant for each allele. Both sequences were represented in cDNAs isolated from the CH5 haustorium cDNA library. To identify further sequence variation at the *AvrP4* locus, RT-PCR was performed on several independent rust strains. An additional sequence variant (*AvrP4*<sup>WA</sup>) was found in rust strain WA, which was isolated from the Australian native flax species *Linum marginale* and is avirulent on flax varieties containing the *P4* resistance gene (Lawrence, 1989).

Each *AvrP4* homolog contains a 285-bp open reading frame encoding a 95-amino acid Cys-rich protein with a predicted 28-amino acid cleavable secretion signal peptide (Figure 4). These

plasmids encoding *AvrM*-A, -B, -C, -D, -E, or *avrM* without the predicted signal peptide. The photograph was taken 4 d after infiltration.



**Figure 4.** Amino Acid Sequence Alignment of the Predicted AvrP4 Proteins.

Only those amino acids that differ from the consensus (top line) are shown, with identical residues indicated by dots. The signal peptide is underlined, and Cys residues and the GFSR motif identified by MEME analysis are shaded in gray.

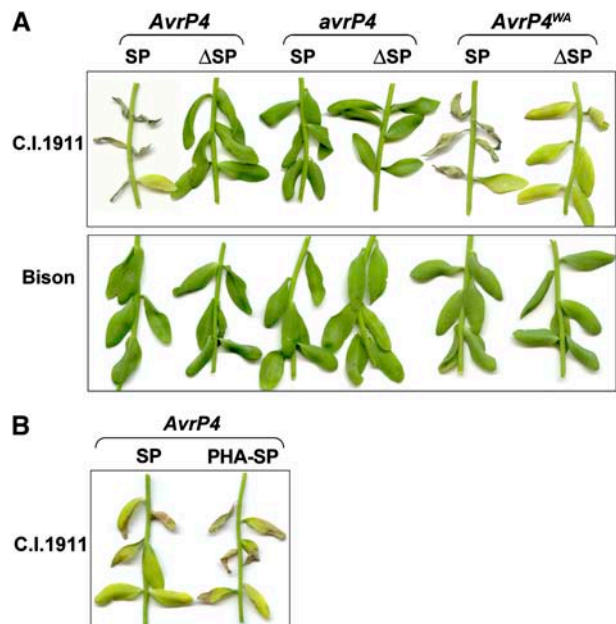
predicted proteins show no significant sequence homology (BLASTP; Altschul et al., 1997) with other proteins and have no predicted motif structures. However, they do contain six Cys residues in the last 28 amino acids of the C terminus that are spaced according to the consensus of an inhibitor Cys knot structure (CX<sub>3-7</sub>CX<sub>4-6</sub>CX<sub>0-5</sub>CX<sub>1-4</sub>CX<sub>4-10</sub>C; Figure 4), which is commonly found in toxins and inhibitors of receptors or proteases (Pallaghy et al., 1994). Within the presumed 67 amino acid mature protein, there are only seven polymorphisms, all of which occur within the last 22 amino acids of the C terminus, a significantly ( $\chi^2$ ;  $P < 2 \times 10^{-4}$ ) nonrandom distribution. None of the six Cys residues represents a polymorphic site.

An *AvrP4* probe was hybridized with the genomic DNA library prepared from the F1 rust CH5 (Ayliffe et al., 2001), and five identical  $\lambda$  clones were isolated containing the *AvrP4* sequence plus 2.4 kb of flanking DNA. Because the alternative allele was not recovered from the genomic DNA library, the corresponding sequence was amplified by long-distance PCR from an F2 rust strain homozygous for this allele. Comparison of the genomic and cDNA sequences revealed two introns of 92 and 86 bp within the 5' untranslated leader of *AvrP4*. The *avrP4* gene contains an additional 5' splice site at the first intron, leading to two alternative transcripts with either 92 or 113 bp removed. Both transcripts were represented at similar levels in the cDNA library and among cloned RT-PCR products and contained an identical open reading frame. A 600-bp region that has been duplicated at the *avrP4* allele begins 1028 bp upstream of the *AvrP4* start codon.

## Transient Expression of *AvrP4* in Flax Causes *P4*-Dependent Cell Death

The *AvrP4* genes were transiently expressed in the flax varieties Bison (which lacks *P4* resistance) and C.I.1911 (which contains *P4*) by *A. tumefaciens* infiltration (Figure 5A). As described above, two sets of T-DNA plasmids were constructed with each gene, one including the entire open reading frame and the other with Met-26 as the first translated codon, which excluded the first 25 amino acids of the 28-amino acid predicted signal peptides. Expression of either *AvrP4* or *AvrP4*<sup>WA</sup>, but not *avrP4*, induced necrosis in C.I.1911 plants but not in Bison, consistent with the predicted avirulence function of these proteins. However, differences were seen between the secreted and nonsecreted ver-

sions. For both *AvrP4* and *AvrP4<sup>WA</sup>*, the full-length construct encoding a secreted protein induced a strong necrotic response. However, truncated *AvrP4<sup>WA</sup>* gave a delayed necrotic response relative to the full-length version, and the truncated *AvrP4* gave no response. The response to the truncated *AvrP4<sup>WA</sup>* construct indicates that recognition of the encoded protein occurs inside



**Figure 5.** Transient Expression of *AvrP4* in Flax Causes *P4* Gene-Dependent Necrosis.

**(A)** Leaves of the flax variety Bison or C.I.1911, which contains *P4*, were infiltrated with *A. tumefaciens* cultures containing T-DNA plasmids encoding *AvrP4*, *AvrP4<sup>WA</sup>*, or *avrP4*. Photographs were taken 10 d after infiltration. Constructs included either the complete transcript, including the signal peptide (SP), or excluded the first 25 amino acids of the 28-amino acid predicted signal peptide, with Met-26 as the first translated codon ( $\Delta$ SP).

**(B)** Leaves of the flax variety C.I.1911 were infiltrated with *A. tumefaciens* cultures containing T-DNA plasmids encoding the native predicted signal peptide (*AvrP4* SP) or a plant signal peptide (*AvrP4* PHA-SP). The photograph was taken 10 d after infiltration.

the plant cell, as observed for AvrM and AvrL567 (Dodds et al., 2004). However, the stronger response observed for the full-length constructs suggests that the presence of the 28-amino acid N-terminal region enhances recognition of the encoded proteins. RNA gel blot analysis on infiltrated flax leaves confirmed that all constructs were expressed at similar levels (data not shown).

As with AvrM, we tested whether the presence of an ER retention signal would inhibit recognition of the secreted AvrP4 protein. However, addition of either the HDEL (ER-retrievable) or HDDL (nonretrievable) motif to AvrP4 abolished recognition (data not shown), indicating that the presence of these additional amino acids interfered with recognition irrespective of ER retention. As an alternative means to determine whether the leader peptide functions as a secretion signal, we replaced the 28-amino acid predicted signal peptides of AvrP4 with the 44-amino acid leader sequence that directs the secretion of bean phytohemagglutinin (PHA) (Tague et al., 1990). Transient expression of *AvrP4 PHA-SP* induced necrosis in C.I.1911 plants but not in Bison (Figure 5B). One likely explanation for the requirement of secretion for full recognition is that the six Cys residues in this protein form disulfide bonds during passage through the oxidizing environment of the ER, which may be important for protein structure and/or stability. Consistent with this hypothesis, mutation of either Cys-84 or Cys-93 to Ala abolished the P4-dependent necrosis-inducing activity of AvrP4 (data not shown). These results suggest that secretion is required for full recognition of the AvrP4 protein expressed in planta, probably because it allows optimal folding and disulfide bond formation.

A yeast signal peptide screen was also used to investigate the predicted signal peptides of AvrM and AvrP4. The nucleotide sequence encoding the first 32 amino acids of both AvrM and AvrP4 was cloned into a yeast shuttle vector upstream of the reporter gene invertase, which lacks its own start codon and signal peptides. The AvrM signal peptide was able to direct secretion of the invertase protein, which was detected as growth on medium containing sucrose as the sole carbon source (see Supplemental Figure 2 online). Although the AvrP4-SP invertase fusion construct did not allow the growth of yeast on sucrose medium, we do not know whether this fusion protein was efficiently expressed, so this negative result is inconclusive and the weight of other evidence suggests that this is a functional signal sequence.

### A Short Conserved Motif Is Present in Flax Rust Avr Proteins

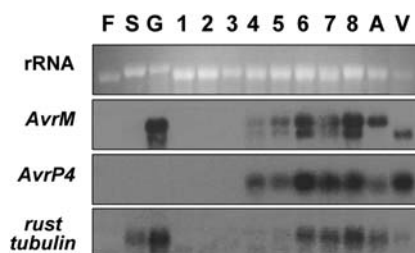
More than 40 predicted oomycete-secreted proteins, including several identified Avr proteins, contain the sequence motif RxLRx<sub>5-21</sub>ddEER (Armstrong et al., 2005; Rehmany et al., 2005), which is related to a host translocation signal in secreted proteins of the malaria parasite (Hiller et al., 2004; Marti et al., 2004). However, the oomycete RxLR motif is absent from all 21 flax rust HESPs (including AvrL567). To search for other conserved motifs, these sequences were analyzed by MEME (Bailey and Elkan, 1994). This analysis identified a short motif in the N-terminal region after the signal peptides of AvrL567 (GYTR), AvrM (GFLR), and AvrP4 (GFSR) and in the C-terminal region of AvrP123 (GIAR). Similar sequences occur in the C-terminal

regions of some of the other HESPs. To examine whether this motif may play a role in the recognition or uptake of these proteins in planta, we generated mutations of this motif in AvrM and AvrP4. All four amino acids were converted to Ala in AvrM and AvrM $\Delta$ SP and also in AvrP4 and AvrP4<sup>W $\Delta$</sup> SP. When transiently expressed in flax plants containing the corresponding *R* genes, these constructs all induced a necrotic response similar to the wild type (data not shown). Therefore, this motif is not important for either recognition of these proteins in the cytoplasm or uptake of the secreted proteins into the cytoplasm during in planta expression.

### AvrM and AvrP4 Have Different Expression Profiles

The expression profiles of *AvrP4* and *AvrM* were examined by RNA gel blot hybridization (Figure 6). Transcripts for *AvrP4* were not detected in RNA samples from rust spores or in vitro-germinated spores, but they were detected in RNA from flax leaves infected with rust strain CH5, indicating that it is induced during infection of the leaf. The increase in abundance from 4 to 8 d after infection probably reflects the increase in fungal biomass during this period. The expression profile of *AvrM* differed from that of *AvrP4*, with *AvrM* transcripts also found in RNA from in vitro-germinated rust spores but not from spore RNA. This finding implies that *AvrM* is induced during germination but before infection of the host has been established and is not dependent on any plant signal (Figure 6). As for *AvrP4*, the greatest amount of transcript was observed in the later stages of infection, when there is a greater fungal biomass. Conversely, the flax rust  $\beta$ -tubulin gene (Ayliffe et al., 2001) is expressed in both germinated and ungerminated spores as well as during infection.

We also examined the expression of *AvrP4* and *AvrM* genes in F2 rust individuals homozygous for either the avirulence or virulence alleles of these loci. Both *AvrP4* and *avrP4* transcripts were detected in RNA samples from rust-infected flax leaves, indicating that both alleles are expressed during infection. Interestingly, the level of expression for *avrP4* appears to be greater



**Figure 6.** *AvrM* but Not *AvrP4* Is Expressed in In Vitro-Germinated Spores.

RNA samples (5  $\mu$ g) from uninfected flax leaves (F), CH5 rust spores (S), in vitro-germinated CH5 rust spores (G), rust CH5-infected flax leaves 1 to 8 d after infection (1 to 8), and rust strains homozygous for either the avirulence (A) or virulence (V) allele (isolated from infected leaves 7 d after inoculation) were separated on a 1.5% agarose gel, which was stained with ethidium bromide to detect rRNA loading (top). RNA from the gel was transferred to a nylon membrane and hybridized with probes for the *AvrM*, *AvrP4*, and  $\beta$ -tubulin genes of *M. lini*.



than that for *AvrP4*. Consistent with this, of nine *AvrP4*-hybridizing clones isolated from the haustorium-specific cDNA library, seven corresponded to *avrP4* and two corresponded to *AvrP4*. These clones represented  $\sim 0.04\%$  of the library. Higher expression of *avrP4* transcripts was also seen in RNA gel blots of the flax leaves infiltrated with the in planta expression constructs for *AvrP4* (data not shown), suggesting that this increased level of message may be an intrinsic feature of the *avrP4* coding sequence. No differences in expression levels from the *AvrM* locus were observed between rust strains homozygous for either the avirulence or the virulence allele. This despite the fact that the avirulence allele contains at least five *AvrM* homologs compared with a single copy at the virulence allele. This observation is supported by the frequency of cDNA clones recovered from the haustorium-specific cDNA library, which included eight of *avrM*, two of *AvrM-A*, two of *AvrM-D*, and one each of *AvrM-B* and *AvrM-E*. Although *AvrM-C* was not identified among these 14 clones, it was recovered by RT-PCR and therefore is expressed. Genes at the *AvrM* locus represent  $\sim 0.15\%$  of clones in the haustorium-specific cDNA library.

### Evidence for Diversifying Selection

Nucleotide variation at the *AvrM* and *AvrP4* loci was analyzed for evidence of positive selection. Among the *AvrM* homologs, most of the amino acid polymorphisms occur between *avrM* and the five *AvrM* proteins. Comparing the nucleotide sequences of *avrM* and the other genes, we found a significant excess of non-synonymous over synonymous changes (ratio = 2.1;  $t$  test,  $P < 0.002$ ). A significant excess of nucleotide substitutions was also found in the coding regions (5 in 1134 bp) relative to the flanking DNA (12 in 9700 bp) when we compared the *AvrM-A*, *-B*, and *-C* genomic sequences ( $\chi^2$  test,  $P < 0.011$ ). These results suggest that selection has contributed to the accumulation of sequence differences between *AvrM* homologs. Although all but one of the nucleotide changes within the coding sequences of the three *AvrP4* genes are nonsynonymous, this difference is not statistically significant. However, a significant excess of nucleotide substitutions was observed in the coding regions (6 in 285) relative to the flanking DNA (11 in 1502) when the *AvrP4* and *avrP4* genomic sequences were aligned ( $\chi^2$ ,  $P < 0.025$ ), suggesting that diversifying selection has acted upon this gene.

### DISCUSSION

The *AvrL567* genes from flax rust, which were previously isolated by map-based cloning, encode secreted proteins and are expressed in haustoria (Dodds et al., 2004). We reasoned that these two features may be characteristic of other Avr proteins in flax rust, because hypersensitive cell death in rust resistance occurs exclusively at sites of haustoria formation (Heath, 1997) and all of the cloned rust resistance genes from flax encode predicted cytoplasmic proteins. Therefore, we searched a haustorium-specific cDNA library for sequences encoding putative secreted proteins. Mapping the identified HESPs in a segregating rust family and in planta expression analysis led to the identification of three additional Avr genes, *AvrM*, *AvrP4*, and *AvrP123*. At the *AvrM* locus, at least five closely related genes segregate with the

avirulence allele in CH5 (*AvrM-A*, *B*, *C*, *D*, and *E*), although it is possible that additional homologs may occur at this haplotype. Genomic sequences flanking three of these genes (*A*, *B*, and *C*) indicate that they are separated by at least 10 to 15 kb. Four of these genes caused *M* gene-dependent cell death when expressed transiently in flax leaves (Figure 3), whereas *avrM*, the single gene at the virulence allele, did not cause necrosis. At the *AvrP4* locus in rust CH5, a single gene is present at each allele. The gene derived from the avirulence allele, *AvrP4*, induced cell death in *P4* plants when transiently expressed in flax leaves, as did a homologous gene (*AvrP4<sup>WA</sup>*) from another rust strain avirulent on *P4*, whereas the virulence allele, *avrP4*, eludes this detection (Figure 5). *AvrP123* is a complex locus in flax rust and encodes avirulence on three resistance genes, *P1*, *P2*, and *P3*. These specificities are tightly linked, but they have been separated by recombination and mutation events in a large-scale screen (Lawrence et al., 1981b). We identified one gene, *AvrP123-A*, that is recognized in planta by the *P1* and *P2* resistance genes (Figure 1). At least three homologs of this gene occur at this locus and may correspond to *P3*.

### Intracellular Recognition of AvrM and AvrP4

When *AvrM* genes were expressed without the signal peptides, a strong *M* gene-dependent necrotic response was observed in flax and tobacco leaves (Figure 3), which implies that recognition of *AvrM* occurs within the plant cell, similar to *AvrL567* (Dodds et al., 2004). This intracellular recognition is consistent with the predicted cytoplasmic location of the NBS-LRR class L and M resistance proteins (Lawrence et al., 1995; Anderson et al., 1997). Interestingly, although the L and M proteins are closely related homologs (75% amino acid identity, 84% similarity), the *AvrL567* and *AvrM* proteins are completely unrelated. As with the L6-*AvrL567* interaction (Dodds et al., 2004), the M-*AvrM* interaction resulted in HR induction in tobacco (Figure 3B). This finding indicates that host factors required for this recognition event and the downstream signaling pathway are sufficiently conserved in tobacco to allow a productive interaction. Expression of *AvrP4<sup>WA</sup>* without its signal peptides also induced *P4* gene-dependent necrosis in flax (Figure 5A), implying that recognition of this protein also occurs inside the plant cell. Although the *P4* resistance gene has not been cloned, the allelic *P* and *P2* genes encode predicted cytoplasmic NBS-LRR proteins (Dodds et al., 2001b), which agrees with an intracellular Avr recognition site. We predict that during infection, *AvrL567*, *AvrM*, and *AvrP4* proteins are secreted from the rust and translocated into the plant cell, where they are recognized by the corresponding cytoplasmic resistance proteins.

### AvrP4 and AvrP123 Are Cys-Rich Proteins

*AvrP4* encode a predicted 67-amino acid protein after cleavage of the signal peptide, with six Cys residues in the last 28 amino acids. These residues comply with the spacing consensus of inhibitor Cys knot structures (Figure 4), such as the *C. fulvum* AVR9 protein (van den Hooven et al., 2001), which form three disulfide bridges. All seven polymorphic residues that distinguish the mature *AvrP4* proteins occur within this C-terminal region,

which may represent a distinct structural domain of the AvrP4 protein. It is possible that this protein may be subject to processing to remove the non-Cys-rich N-terminal region, similar to the processing of Avr9 (van den Ackerveken et al., 1993). The SIX1 protein of *F. oxysporum*, which determines avirulence on the *I-3* resistance gene in tomato, is also a Cys-rich protein that is subject to posttranslational processing. In this case, a 12-kD protein containing six Cys residues occurs in xylem sap of infected plants and corresponds to the central region of the predicted 30-kD precursor protein (Rep et al., 2004). Given these considerations, passage of AvrP4 through the oxidizing environment of the ER during secretion may be necessary for efficient folding and formation of disulfide bonds or posttranslational processing. Indeed, mutation of two of these residues, Cys-84 and Cys-93, caused a loss of recognition, which would be expected if these were involved in structurally important disulfide bonds. Similarly, altered Cys residues in some virulence alleles of the *C. fulvum* AVR4 protein lead to instability of these proteins as a result of the disruption of an internal disulfide bond (Joosten et al., 1994). This hypothesis would explain the requirement for the signal peptides for full recognition of AvrP4 and the stronger response induced by AvrP4<sup>WA</sup> when the signal peptide is retained (Figure 5A). In this situation, the weaker recognition of AvrP4<sup>WA</sup>ΔSP may be attributable to the presence of a small amount of correctly folded AvrP4<sup>WA</sup> in the cytoplasm. The difference between the responses induced by the AvrP4ΔSP and AvrP4<sup>WA</sup>ΔSP proteins may reflect either their relative efficiency of folding in the cytoplasm or intrinsic differences in recognition strength. Because the PHA signal peptide was able to substitute for the N-terminal 28 amino acids of AvrP4 to allow recognition (Figure 5B), it is likely that the requirement for this region relates directly to its role in secretion.

AvrP123-A is also a Cys-rich protein and contains a signature (CX<sub>7</sub>CX<sub>6</sub>YX<sub>3</sub>CX<sub>2-3</sub>C; Figure 1A) characteristic of the Kazal family of Ser protease inhibitors. Two extracellular Kazal-like Ser protease inhibitors from the oomycete *P. infestans* were found to inhibit the pathogenesis-related P69B protease in tomato (Tian et al., 2004, 2005). Interestingly, previous database searching (Tian et al., 2004) identified 35 predicted proteins with Kazal motifs from oomycetes but none from fungi. The oomycete Kazal proteins generally contain an Asp residue at the P1 position (Tian et al., 2004), which is important in determining protease interaction specificity, whereas the corresponding residue in AvrP123-A is a Cys. Thus, it is possible that AvrP123 proteins may be inhibitors of host proteases, as is also observed for the Avr2 protein from *C. fulvum*, which inhibits the Cys protease Rcr3 in tomato (Rooney et al., 2005).

### The Role of Sequence Variation in AvrM and AvrP4 Recognition

Positive selection appears to have contributed to the accumulation of sequence differences within the coding sequences of the *AvrP4* and *AvrM* genes. Genes at both loci show an excess of nucleotide differences in the coding region relative to the flanking genomic DNA, and we found a significant excess of nonsynonymous over synonymous substitutions that distinguish the virulence allele *avrM* from *AvrM* genes at the avirulence allele. In the

case of *avrM*, lack of recognition may be attributable to the 10 unique polymorphic amino acids that are concentrated in the C-terminal region (Figure 2B) or to the 69-residue internal deletion in the N-terminal region. As high levels of transcript from this gene can be found in both avirulent and virulent homozygotes, escaping recognition does not appear to be caused by an absence or low rate of gene expression, although we do not know whether the mRNA is translated to a stable protein. Of the five *AvrM* genes from the avirulence allele, the only one to elude detection is *AvrM-E*, which encodes a truncated protein lacking 131 amino acids from its C terminus (Figures 2A and 2B). The absence of this region may prevent recognition or lead to protein instability. The presence of a 34-amino acid C-terminal extension in some *AvrM* proteins (Figures 2A and 2B) also has some effect on recognition, because *AvrM-A* and *-D*, which lack this region, gave a stronger response than *AvrM-B* and *-C*.

Differences in recognition between AvrP4 proteins apparently result from the amino acid differences in the C-terminal Cys-rich domain (Figure 4), suggesting that this region is important for recognition. Furthermore, AvrP4 recognition was abolished when additional amino acids (HDEL and HDDL) were added to the C terminus of this protein, further supporting the involvement of the C-terminal region in recognition. Again, the *AvrP4* and *avrP4* alleles are both expressed at the mRNA level, with *avrP4* transcripts actually more abundant. Thus, escaping recognition does not appear to be attributable to an absence of gene expression, although, as for *AvrM*, we do not know whether the translated protein is stable and able to accumulate. As mentioned above, the generation of unstable AVR4 isoforms allows virulent strains to avoid Cf-4 recognition (Joosten et al., 1994, 1997), whereas, conversely, *C. fulvum* strains virulent on plants carrying Hcr9-4E can evade detection by producing a stable Avr4E protein with only two amino acid substitutions (Westerink et al., 2004). Evidence for selection underlying amino acid variation was also found between the AvrL567 proteins (Dodds et al., 2004) and the *H. parasitica* ATR13 and ATR1<sup>NdWsB</sup> proteins (Allen et al., 2004; Rehmany et al., 2005). In all cases, selection imposed by the presence of corresponding *R* genes in the host appears to be the driver for the accumulation of Avr variation, leading to escape from recognition.

### Translocation of Avirulence Proteins into Host Cells

The identification of three flax rust avirulence loci that encode secreted proteins expressed in haustoria and recognized inside plant cells supports the hypothesis that, like bacterial pathogens, rust fungi secrete a suite of effector proteins into infected host cells. These proteins are likely to have important roles in pathogenesis-related processes. However, the mechanism of translocation from the extrahaustorial matrix across the plant cell membrane is not known. We consider there to be two main possibilities for this process. It may be mediated by a specialized translocation apparatus produced by the rust haustorium, or it may rely on intrinsic plant cross-membrane transport mechanisms. The *AvrM-B* and *-C* proteins induced a weaker necrotic response when secreted (data not shown), as did the AvrL567 proteins recognized by L6 (Dodds et al., 2004). Although this is consistent with the hypothesis that these proteins may reenter

the cell after secretion in the absence of the rust pathogen, these observations could be explained by the accumulation of small amounts of these proteins in the cytoplasm as a result of aberrant processing of the signal peptide or inefficient secretion. However, the secreted versions of AvrM-A and -D induce a very strong necrotic response, which in the case of AvrM-A is equivalent to the nonsecreted version (data not shown), which is more difficult to explain on this basis. Furthermore, the addition of an ER retrieval signal to the full-length AvrM-A protein inhibited the necrotic response (Figure 3B), indicating that this protein is directed through the secretory pathway in planta and that the secreted version is able to enter the host cytoplasm from the apoplast in the absence of the pathogen. Addition of the HDEL motif to a secreted Avr9 protein also abolished Cf-9-Avr9-dependent necrosis (Wilson et al., 2005). Similarly, as discussed above, it is likely that the much stronger recognition of the secreted AvrP4 proteins compared with the nonsecreted versions is the result of the more efficient formation of disulfide bonds in the ER during secretion. Thus, the enhanced recognition would be conferred by correctly folded AvrP4 protein that has passed through the secretory pathway and then reentered the cell. The question remains whether this observation represents an experimental artifact that may be caused by artificially high levels of these proteins in the transient assay system, or do these rust proteins hijack a plant-derived translocation mechanism to gain entry to host cells?

Several avirulence products identified from oomycetes are also thought to be recognized after transport into the host cell. Like rusts, these pathogens grow intercellular hyphae and form haustoria that are surrounded by a plant-derived plasma membrane. The secreted protein ATR13 from *H. parasitica* triggers RPP13 resistance in *Arabidopsis thaliana* when transiently expressed in planta without its signal peptide (Allen et al., 2004). This intracellular recognition implies that ATR13 is transported into the plant cell during infection. A similar situation was observed with ATR1<sup>NdW<sup>SB</sup></sup> also from *H. parasitica* (Rehmany et al., 2005) and Avr3a from *P. infestans* (Armstrong et al., 2005). In each case, the R protein responsible for recognition of the Avr elicitor is a cytoplasmic NBS-LRR protein (Botella et al., 1998; Allen et al., 2004; Armstrong et al., 2005). Therefore, the predicted localization of the R protein is consistent with the Avr recognition site. Similar to AvrM-A and -D, the presence or absence of the signal peptides for the ATR13 avirulence product from *H. parasitica* did not affect its activity in transient expression analysis in planta (Allen et al., 2004), whereas a reduced response was observed with the inclusion of the signal peptides for Avr3a and ATR1<sup>NdW<sup>SB</sup></sup> (Armstrong et al., 2005; Rehmany et al., 2005). In another example, infiltration of recombinantly expressed Avr1b-1 protein into the apoplast of soybean (*Glycine max*) cultivars containing the *Rps1b* resistance gene resulted in necrosis, suggesting that this secreted Avr protein from *P. sojae* may enter the plant cell in the absence of the pathogen (Shan et al., 2004), although the site of recognition has not been confirmed.

The oomycete Avr proteins all contain a sequence motif (RxLR<sub>x<sub>5-2</sub></sub>ddEER) (Armstrong et al., 2005; Rehmany et al., 2005) that is similar to the host-targeting signal (RxLx<sub>E</sub>/Q) that directs the transport of secreted malaria proteins across the

parasitophorous vacuolar membrane into the cytoplasm of erythrocytes (Hiller et al., 2004; Marti et al., 2004). This similarity suggests that the oomycete motif may also be involved in the translocation of proteins into the host plant cell. This motif is also present in at least 40 other predicted oomycete-secreted proteins and may define a set of translocated effector proteins in these pathogens; interestingly, this motif appears to be absent from secreted Cys-rich proteins (Rehmany et al., 2005). The oomycete RxLR motif is not present in the flax rust Avr proteins, suggesting that secreted rust effector proteins may use a different translocation mechanism. We did identify a short motif (Gx<sub>1</sub>x<sub>2</sub>R, where x<sub>1</sub> is a hydrophobic or aromatic amino acid) in the N-terminal region of AvrL567, AvrM, and AvrP4 and in the C-terminal region of AvrP123, which may represent a motif common to rust avirulence proteins. Mutations of this motif did not affect the recognition of either secreted or nonsecreted versions of AvrM or AvrP4 expressed in planta. Although this indicates that this motif is not important for the uptake of the plant-produced secreted proteins, we cannot exclude a role in the translocation of rust-derived proteins during infection.

It is noteworthy that a relatively modest sequencing effort of only 672 ESTs yielded 21 HESPs that included four confirmed avirulence genes: *AvrL567*, *AvrM*, *AvrP4*, and *AvrP123*. It is possible that some of the other HESPs may also encode avirulence genes, because only 16 of the >30 known avirulence specificities segregate in the CH5-derived mapping family. Thus, this approach of screening haustorium ESTs for those encoding predicted signal peptides has proven to be a powerful method to isolate Avr genes from flax rust. It is likely that this approach will also be applicable to other rust species that cause severe disease on crops such as soybean and wheat (*Triticum aestivum*). It is likely that rust HESPs include proteins with important pathogenicity functions, and further study of these genes may provide insights into the parasitic interaction between rust fungi and their hosts.

## METHODS

### Rust and Plant Material

A flax rust (*Melampsora lini*) F2 family derived from selfing rust strain CH5, the F1 hybrid of a cross between parental strains C and H, was described by Lawrence et al. (1981a). The B12×M, B10×P1, B4×P2, and B13×P3 flax (*Linum usitatissimum*) lines contain the *M*, *P1*, *P2*, and *P3* resistance genes backcrossed for 12, 10, 4, or 13 generations, respectively, into the variety Bison (Lawrence et al., 1981a). The flax line C.I.1911, which contains *P4*, was described by Zimmer and Comstock (1973). A flax line containing the *M* transgene in the variety Hoshangabad was described by Anderson et al. (1997), and the same *M* gene construct was transformed into the tobacco (*Nicotiana tabacum*) line W38 as described by Frost et al. (2004). Rust inoculations were performed as described by Lawrence et al. (1981a). Rust spores were germinated overnight on water, and genomic DNA was isolated from the resulting mycelial mat as described by Anderson et al. (1997).

### Isolation of Flax Rust Haustorial Cells

Haustoria were isolated from flax leaves of the rust-susceptible variety Hoshangabad at 6 d after inoculation with rust strain CH5 by affinity chromatography essentially as described by Hahn and Mendgen (1992).

A 100- $\mu$ m pore size nylon mesh was used to remove the bulk of the plant cell material from the crude preparation, which was then passed through an 11- $\mu$ m pore size nylon mesh to remove intact plant cells. An affinity column was prepared by covalently attaching concanavalin A (Pharmacia Biotech) to cyanogen bromide-activated Sepharose 6MB (Pharmacia Biotech) as described in the manufacturer's protocol. Samples of purified haustoria were examined by immunofluorescence labeling with monoclonal antibody ML1 as described by Murdoch et al. (1998), except that samples were fixed to slides in 0.5% gelatin, 0.05% chromic potassium sulfate, and 0.02%  $\text{NaN}_3$  and the secondary antibody used was the goat anti-mouse coupled to the fluorophore Alexa 488 (Molecular Probes). Slides were examined on a Leica TCS SP2 confocal microscope under light or under excitation by an argon ion laser at a wavelength of 488 nm. Images were collected at 500 to 530 nm with pseudocolor green.

### Haustrum-Specific cDNA Library and Sequence Analysis

Total RNA was isolated from frozen haustoria samples using the Qiagen Plant RNeasy kit, then treated with DNase and reisolated on the Qiagen Plant RNeasy column. A cDNA library was prepared from 1  $\mu$ g of total RNA from haustoria using the SMART cDNA library kit (Clontech) according to the manufacturer's instructions. Library clones were colony-blotted and then probed with a subset of flax rust rRNA genes as described below, and positives were eliminated before sequencing. DNA sequence data were obtained from PCR cycle sequencing using the enzymatic dideoxy chain-termination method with an ABI PRISM BigDye primer cycle sequencing kit. BLAST searches were made at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>). Searches for protein signatures were performed by Motif Scan ([http://myhits.isb-sib.ch/cgi-bin/motif\\_scan](http://myhits.isb-sib.ch/cgi-bin/motif_scan)). The *AvrM* and *AvrP4* coding sequences or flanking sequences were aligned, and nucleotide sequence distances were calculated across all sites or specifically for nonsynonymous or synonymous sites using the Jukes-Cantor algorithm of the Molecular Evolutionary Genetics Analysis software version 1.02 (<http://evolgen.biol.metro-u.ac.jp/MEGA/>). The significance of differences between average pairwise nucleotide distances was assessed by *t* test.

### Colony and Plaque Lifts and Gel Blot Analysis

Plaques or colonies from the haustorium-specific cDNA library or plaques from a  $\lambda$  EMBL3 genomic DNA library prepared from rust strain CH5 (Ayliffe et al., 2001) were transferred to Hybond  $\text{N}^+$  nylon membranes (Amersham). Restriction enzyme-digested genomic DNA was separated on 1.0% agarose gels and transferred to Hybond  $\text{N}^+$  nylon membranes. RNA samples were separated on 1.5% agarose gels and transferred to Hybond  $\text{N}^+$ . Prehybridization and hybridization with [ $^{32}\text{P}$ ]dCTP-labeled DNA probes were performed in 7% (w/v) SDS, 1% (w/v) BSA, 0.5 M sodium phosphate, pH 7.2, and 1 mM EDTA at 65°C, and washing was in  $1\times$  SSC (0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at 65°C.

### Transcript Analysis by RT-PCR

Total RNA was reverse-transcribed using Superscript reverse transcriptase (Gibco BRL) with an oligo(dT<sub>25</sub>) primer and then amplified by Taq polymerase with the following thermal profile: 94°C for 2 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 to 2 min; 72°C for 5 min. *AvrM* transcripts were amplified with the primers F1-C29 (5'-GGTACCTACCTTTTGAACC-3') and R1-C29 (5'-CGCATCCAAGAAATCACAAATC-3') or FA-C29 (5'-CCACGGAGATAGCTTGTC-3') and Rstop-C29 (5'-GCTTTCAAGGTTTGTCTAATG-3'). *AvrP4* transcripts were amplified with F3-C48 (5'-CATCAAAATCTAACCCTGAC-3') and R1-C48 (5'-GTAGCATTTGAGATCCATGG-3'). RT-PCR products were cloned into pGEM T-Easy, and independent clones were sequenced.

### Transient in Planta Expression Assays

Gene expression constructs contained the *AvrM*, *AvrP4*, and *AvrP123* coding sequences inserted between a 35S cauliflower mosaic virus promoter and a nopaline synthase terminator in the binary vector pTNotTReg (Anderson et al., 1997). The *AvrM* and *AvrP123* expression constructs were prepared by inserting a cloned PCR product as an *EcoRI* fragment. Constructs encoding the truncated *AvrM* proteins, lacking the signal peptide, begin at nucleotide position 79 relative to the first ATG and have a GGA-to-ATG alteration at positions 82 to 84 to incorporate a start Met at amino acid 28, the last amino acid of the predicted signal peptide. Constructs encoding the full-length *AvrM* proteins contain the entire coding sequences as well as four nucleotides 5' to the first ATG. The construct encoding the full-length *AvrP123* protein contains the entire coding sequences as well as 87 nucleotides 5' to the first ATG and 194 nucleotides of the 3' untranslated region. The *AvrP4* expression constructs were constructed by inserting a cloned PCR product as a *BamHI*-*SacI* fragment. Constructs encoding the truncated *AvrP4* proteins begin at nucleotide position 49 relative to the first ATG, making the start of the open reading frame at Met-26, the third last amino acid of the 28-amino acid predicted signal peptide. Constructs encoding the full-length *AvrP4* proteins contain the entire coding sequences as well as 22 nucleotides 5' to the first ATG. Cys-to-Ala mutations were generated by PCR amplification using a 3' primer to incorporate GCT at codon position 84 or 93. The constructs containing the *PHA* signal peptides have the *AvrP4* gene sequence starting at nucleotide position 85 relative to the first ATG plus a *BamHI* fragment encoding the first 44 amino acids of the bean *PHA* (Tague et al., 1990). Between the *PHA* and *AvrP4* sequences are the additional residues Gly-Ser, resulting from the *BamHI* cloning site. *AvrM*-A and *AvrP4* constructs encoding an ER retrieval signal contain 12 additional bases (CATGATGAGCTC, encoding HDEL, or CATGATGACCTC, encoding HDDL) inserted immediately before the stop codon. Quadruple Ala mutants in the GFLR and GFSR motifs of *AvrM*-A and *AvrP4* were generated by recombinant PCR amplification. All constructs were fully sequenced to confirm their integrity. *Agrobacterium tumefaciens* cultures of strain GV3101 containing the binary vector expression constructs were prepared at an OD<sub>600</sub> of 1.0 in liquid Murashige and Skoog medium containing 200  $\mu$ M acetosyringone and infiltrated into flax or tobacco leaves by syringe.

### Yeast Signal Peptide Screen

The nucleotide sequences encoding the first 28 amino acids of *AvrM* or *AvrP4* were cloned as an *EcoRI*-*NotI* fragment into the yeast shuttle vector pSMASH V2, which contains *ura3* as a selectable marker (Goo et al., 1999). Constructs were transformed into the yeast strain DBY $\alpha$ 2445, and cells were grown on yeast minimal medium with sucrose in place of glucose. The pSMASH V2 vector containing the invertase signal peptides or just a start Met were used as positive or negative controls, respectively. The 44-amino acid leader sequence of bean *PHA* was also used as a positive control.

### Accession Numbers

Sequence data from this article can be found in the GenBank data library under accession numbers DQ279864 to DQ279890.

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Transient Expression of the Full-Length *AvrM* Genes in Flax.

**Supplemental Figure 2.** Activity of *AvrM* and *AvrP4* Signal Peptides in Yeast.

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