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Differential Recognition of Highly Divergent Downy Mildew Avirulence Gene Alleles by *RPP1* Resistance Genes from Two Arabidopsis Lines

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The perception of downy mildew avirulence (*Arabidopsis thaliana Recognized* [*ATR*]) gene products by matching *Arabidopsis thaliana* resistance (*Recognition of Peronospora parasitica* [*RPP*]) gene products triggers localized cell death (a hypersensitive response) in the host plant, and this inhibits pathogen development. The oomycete pathogen, therefore, is under selection pressure to alter the form of these gene products to prevent detection. That the pathogen maintains these genes indicates that they play a positive role in pathogen survival. Despite significant progress in cloning plant *RPP* genes and characterizing essential plant components of resistance signaling pathways, little progress has been made in identifying the oomycete molecules that trigger them. Concluding a map-based cloning effort, we have identified an avirulence gene, *ATR1^{NdWsB}*, that is detected by *RPP1* from the Arabidopsis accession Niederzenz in the cytoplasm of host plant cells. We report the cloning of six highly divergent alleles of *ATR1^{NdWsB}* from eight downy mildew isolates and demonstrate that the *ATR1^{NdWsB}* alleles are differentially recognized by *RPP1* genes from two Arabidopsis accessions (Niederzenz and Wassilewskija). *RPP1-Nd* recognizes a single allele of *ATR1^{NdWsB}*; *RPP1-WsB* also detects this allele plus three additional alleles with divergent sequences. The Emco5 isolate expresses an allele of *ATR1^{NdWsB}* that is recognized by *RPP1-WsB*, but the isolate evades detection in planta. Although the Cala2 isolate is recognized by *RPP1-WsA*, the *ATR1^{NdWsB}* allele from Cala2 is not, demonstrating that *RPP1-WsA* detects a novel *ATR* gene product. Cloning of *ATR1^{NdWsB}* has highlighted the presence of a highly conserved novel amino acid motif in avirulence proteins from three different oomycetes. The presence of the motif in additional secreted proteins from plant pathogenic oomycetes and its similarity to a host-targeting signal from malaria parasites suggest a conserved role in pathogenicity.

INTRODUCTION

Plant-pathogenic oomycetes are responsible for economically and environmentally devastating epidemics, including the 19th century Irish potato famine (*Phytophthora infestans*) and the current sudden oak death (*Phytophthora ramorum*) epidemic in California. The obligate biotrophic oomycete *Hyaloperonospora parasitica* (formerly *Peronospora parasitica* [Constantinescu and Fatehi, 2002]) causes downy mildew of the model plant *Arabidopsis thaliana*. Arabidopsis accessions that are resistant to *H. parasitica* carry *Recognition of Peronospora parasitica* (*RPP*) resistance genes. RPP proteins perceive avirulence (*Arabidopsis thaliana Recognized* [*ATR*]) gene products produced by *H. parasitica* isolates and trigger resistance responses. Coevolving populations of Arabidopsis and *H. parasitica* have been used to genetically define and clone Arabidopsis *RPP* genes that represent two intracellular classes and an extracellular class of Leu-rich repeat disease resistance genes (Holub et al., 1994; Parker et al., 1997; Botella et al., 1998; McDowell et al., 1998; Bittner-Eddy et al., 2000; van der Biezen et al., 2002; Sinapidou et al., 2004; Tör et al., 2004). Studying the interaction between RPP proteins and their *H. parasitica* targets represents an opportunity to examine the mechanisms underlying host resistance to, and parasite pathogenicity of, a naturally occurring parasite of the model plant Arabidopsis. To analyze this interaction in both plant and pathogen, it is necessary to identify the *ATR* genes complementary to specific *RPP* genes. These can then be used as tools to investigate their role in pathogenicity and to assess the role and strength of natural selection at the molecular level in host–parasite interactions.

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Few avirulence genes have been cloned from oomycetes or obligate biotrophs (i.e., plant pathogens that cannot be cultured in vitro because their life cycle is entirely dependent on living host tissue). *ATR13* from *H. parasitica* was cloned by isolating an in planta–expressed sequence that cosegregated with and encoded ATR13 (Allen et al., 2004). By contrast, map-based cloning approaches resulted in the identification of *Avr1b-1* from *Phytophthora sojae* (Shan et al., 2004) and *AvrL567* from *Melampsora lini* (Dodds et al., 2004). All three genes are predicted to encode proteins with N-terminal signal peptides, suggesting that they are secreted from the pathogens. Alleles of *ATR13* and *AvrL567* encode proteins with extreme levels of amino acid polymorphism, which is indicative of diversifying selection and potentially driven by, and driving, the polymorphism seen in the matching resistance proteins (Dodds et al., 2000, 2004; Allen et al., 2004; Rose et al., 2004). Alleles of *Avr1b-1* are also polymorphic, and some virulent *P. sojae* isolates carry avirulence *Avr1b-1* alleles but avoid detection because *Avr1b-1* mRNA fails to accumulate in these isolates (Shan et al., 2004).

Genes at the *RPP1* locus of different Arabidopsis accessions recognize different but overlapping sets of *H. parasitica* isolates (Holub et al., 1994; Botella et al., 1998). In accession Wassilewskija (Ws-0), four genes at the *RPP1* locus have been described: *RPP1-WsA* specifies resistance to *H. parasitica* isolates Emoy2, Maks9, Noks1, and Cala2; *RPP1-WsB* detects Emoy2, Maks9, and Noks1; *RPP1-WsC* detects Noks1 alone; and *RPP1-WsD* was not studied (Botella et al., 1998) (Table 1). Botella and coworkers (1998) speculated that, although the *RPP1-Ws* genes may recognize allelic avirulence gene determinants, it was more likely that they detected different avirulence gene products. A single functional *RPP1* gene in accession Niederzenz (Nd-1) specifies resistance to the *H. parasitica* isolates Emoy2, Hiks1, and Waco5 but not Maks9 (Gordon, 2002). The overlapping recognition profiles of *RPP1* genes from the two accessions are intriguing; we are interested in elucidating the complex interaction between these genes and their matching *ATR* gene(s).

ATR1Nd, the avirulence gene perceived by the resistance gene *RPP1* carried by the Arabidopsis accession Nd-1, segregates as a single dominant locus in an F2 mapping population derived from a cross between *H. parasitica* isolates Emoy2 (avirulent) and Maks9 (virulent). A mapping interval spanning *ATR1Nd* was defined, and a contig spanning the genetic interval was assembled using clones from a BAC library constructed from isolate Emoy2 (Rehmany et al., 2003). We have used the sequence across the genetic interval to identify *ATR1Nd*, described here, and to analyze a conserved syntenic region in *P. infestans* (Armstrong et al., 2005). ATR1Nd is a secreted protein that is recognized in the host cytoplasm and is under intense diversifying selection. As well as a signal peptide, it contains a novel motif conserved in many secreted oomycete proteins. Furthermore, our analysis reveals that (1) RPP1-Nd recognizes a single allelic form of *ATR1Nd*; (2) RPP1-WsB recognizes highly variable allelic forms of the gene; (3) RPP1-WsA does not detect this gene in the *H. parasitica* isolate Cala2; and (4) the isolate Emco5 expresses a form of *ATR1Nd* that RPP1-WsB is capable of detecting, but detection does not occur when the pathogen expresses the gene during infection.

RESULTS

A Candidate for *ATR1Nd* Encodes a Polymorphic Secreted Protein

The sequence of the genetic interval containing *ATR1Nd* (Rehmany et al., 2003) was used to identify new genetic markers to refine the interval further. One marker revealed five single nucleotide polymorphisms (SNPs) between Emoy2 and Maks9 that were found to cosegregate with *ATR1Nd* in the F2 mapping population of 311 F2 individuals. Furthermore, the SNPs were within a region that was predicted to be protein-coding by an algorithm differentiating the base composition of coding and noncoding DNA sequences (Fickett, 1982). The predicted protein-coding sequence resided between nucleotides 17,975 and 18,930 (numbers relate to BAC clone 12I13). An open reading frame (ORF) starting with ATG was identified (nucleotides 17,977 to 18,909); within this ORF, all five SNPs between the Emoy2 and Maks9 sequences were predicted to result in nonsynonymous amino acid substitutions (Figures 1 and 2). The ORF was predicted to encode a protein with a signal peptide ($P = 0.99$; SignalP) (Bendtsen et al., 2004); therefore, the protein would appear to be secreted, exposing it to plant detection mechanisms and consistent with two previously reported

Table 1. Differential Resistance Phenotypes Determined by *RPP1* Loci from Nd-1 and Ws-0

H. parasitica Isolate	Nd-1	Ws-0	Resistance Specified by *RPP1-Ws* Genes[a]
Emoy2	Resistant[b]	Resistant	*RPP1-WsA*, *RPP1-WsB*
Hiks1	Resistant[b]	Resistant	NT
Waco5	Resistant[b]	Resistant	NT
Maks9	Susceptible	Resistant	*RPP1-WsA*, *RPP1-WsB*
Noks1[c]	Susceptible	Resistant	*RPP1-WsA*, *RPP1-WsB*, *RPP1-WsC*
Cala2	Susceptible	Resistant	*RPP1-WsA*
Emco5	Susceptible	Susceptible	NT
Emwa1	Susceptible	Susceptible	NT

[a] Individual *RPP1-Ws* genes are sufficient to provide resistance to the *H. parasitica* isolates shown (Botella et al., 1998). NT, not tested.
[b] *RPP1* locus resistance specified by a single gene, *RPP1-Nd* (Gordon, 2002).
[c] Noks1 was derived from an oospore of Noco2 (used in Botella et al., 1998), and its interaction phenotype matches that of Noco2 (Holub et al., 1994).

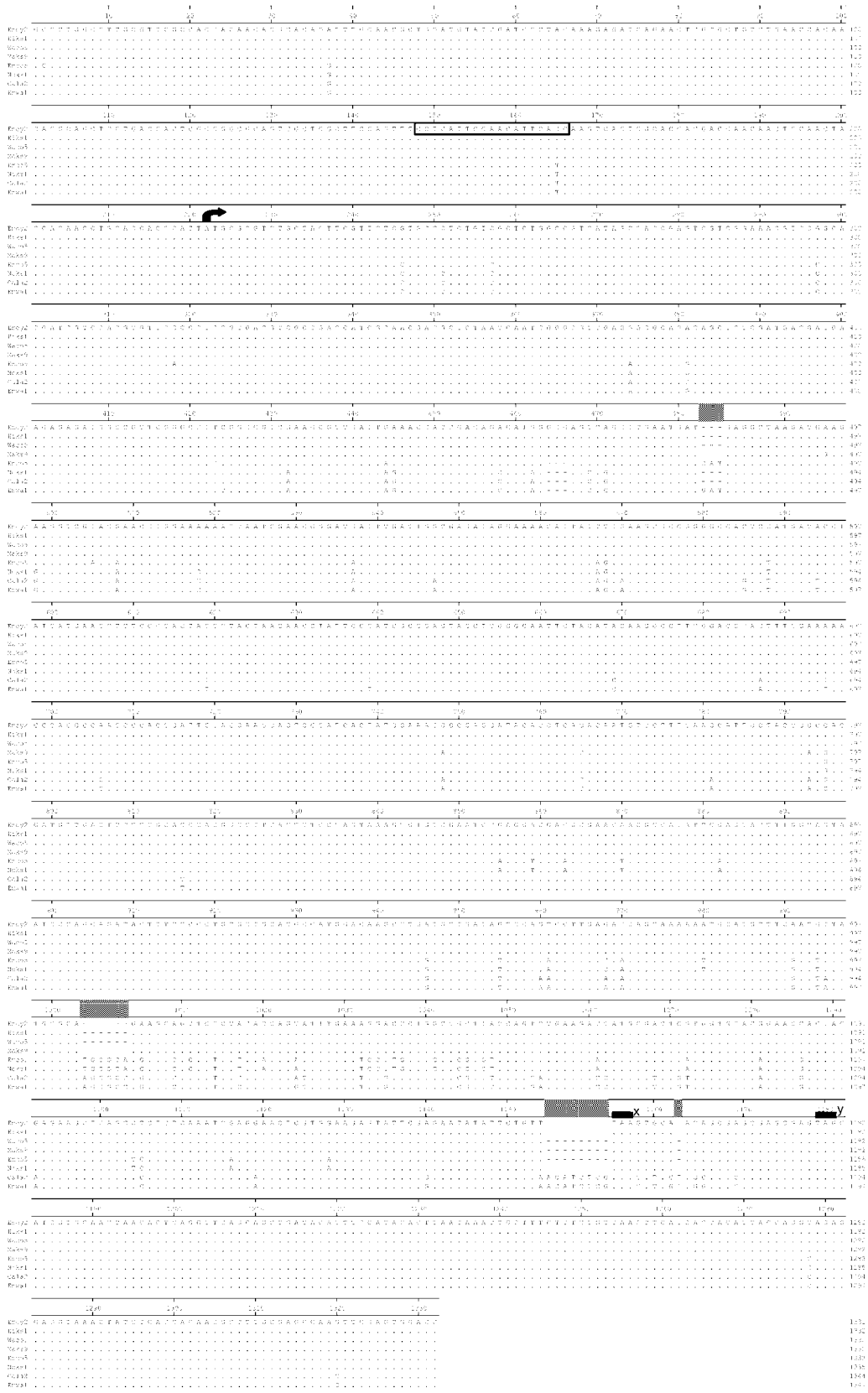

Figure 1. Alignment of *ATR1*^{NdWsB} Allele Sequences, Including Flanking DNA, from Eight *H. parasitica* Isolates.

Dots indicate nucleotides identical to the Emoy2 sequence; dashes indicate gaps in the alignment. The predicted transcription initiation sequence (boxed), translational start (arrow), and stop (rectangles; x for Emoy2, Hiks1, Waco5, Maks9, Emco5, and Noks1; y for Cala2 and Emwa1) codons are marked. Nucleotide numbers, above the alignment, correspond to the Emoy2 allele and to nucleotides 17,756 to 19,087 of BAC 12I13.

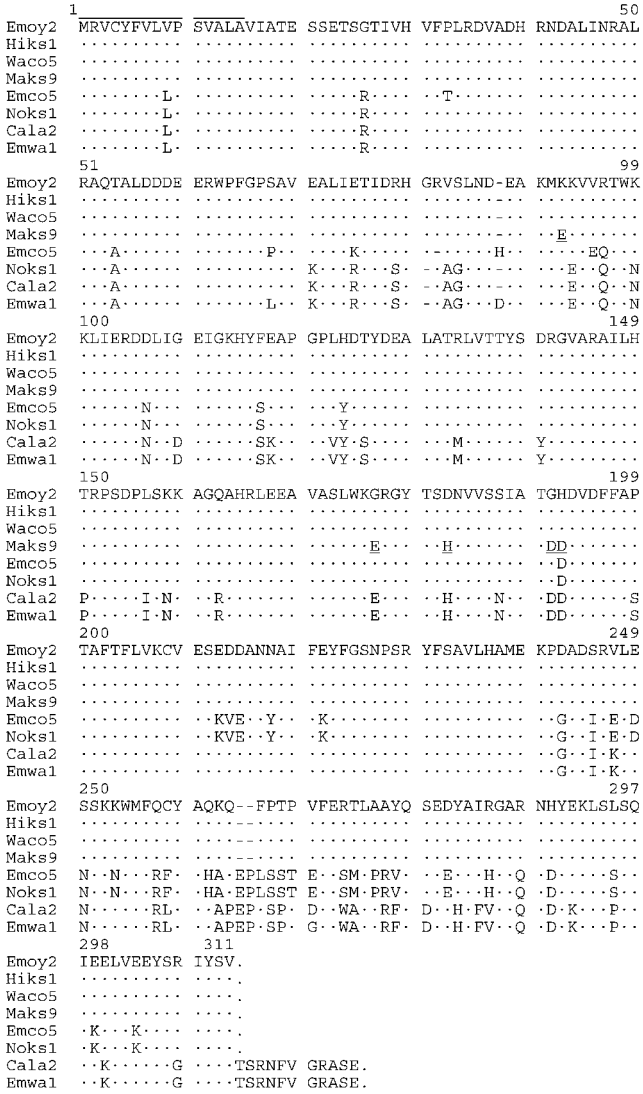

Figure 2. Alignment of Predicted ATR1^{NdWsB} Proteins from Eight *H. parasitica* Isolates.

Dots indicate amino acids identical to the Emoy2 sequence; dashes indicate gaps in the alignment; full stops indicate stop codons. The predicted N-terminal signal peptide is marked with a line above the Emoy2 sequence. All five polymorphisms between Emoy2 and Maks9 are underlined in the Maks9 sequence. Amino acid numbers, above the alignment, correspond to the Emoy2 sequence.

oomycete avirulence genes (Allen et al., 2004; Shan et al., 2004). Other than the signal peptide, the encoded protein had no recognizable functional motifs, and no related sequences were found in public databases. A sequence (CCTCATTCCAACATTCACC) sharing 15 of 19 identical nucleotides with an oomycete consensus transcription initiation sequence (GCYCA$_{+1}$TTYYNTTYYNCAWTTTNYY [McLeod et al., 2004]) was observed upstream of the predicted start (ATG) codon (nucleotides −74 to −56; Figure 1); rapid amplification of cDNA ends experiments indicated that the transcription start site of the gene was −70 nucleotides upstream of the start codon, corresponding to the first A of the putative transcription initiation sequence (data not shown). Primers corresponding to the ORF amplified products from cDNA derived from plant material infected with Emoy2 or Maks9; therefore, the gene is expressed during infection (data not shown). The sequence from the cDNA was identical to the BAC sequence, indicating that the ORF contains no introns.

The ORF and flanking region, corresponding to 221 bp upstream and 175 bp downstream of the Emoy2 ORF, were sequenced from eight *H. parasitica* isolates (Table 1, Figure 1). All alleles encoded full-length proteins, and none displayed substitutions rendering them obvious null alleles. Isolates Hiks1 and Waco5 carried the same allele (i.e., identical at the nucleotide level) as Emoy2, which was used in the mapping study; the alleles from the other five isolates differed from the Emoy2 allele and from each other. The six different alleles encoded proteins with very high levels of amino acid polymorphism (Figure 2). The average pairwise differences among alleles at nonsynonymous sites, $\pi = 0.0599$, greatly exceed that at synonymous sites, $\pi = 0.0164$. These alleles show 90 nonsynonymous and only 9 synonymous segregating polymorphisms. Based on a total of 720.67 nonsynonymous sites and 216.83 synonymous sites, this represents a significant excess of nonsynonymous polymorphism relative to the neutral expectation ($\chi^2 = 10.94$, $P = 0.0009$) and indicates selective maintenance of amino acid polymorphism at this locus.

Interestingly, isolates avirulent with RPP1-Nd (Emoy2, Hiks1, and Waco5) had identical DNA sequences, whereas virulent isolates (Maks9, Noks1, Cala2, Emco5, and Emwa1) showed highly divergent DNA sequences within the ORFs and predicted protein sequences (Figures 1 and 2). Because the gene was predicted to encode a secreted protein that genetically cosegregates and shows allelic correlation with the phenotype conferred by *ATR1Nd*, it was a candidate for *ATR1Nd*.

Transient Expression of *ATR1Nd* Causes RPP1-Nd–Dependent Cell Death

A transcriptional fusion was made between the *uidA* gene (β-glucuronidase [GUS]) and the 35S promoter. Full-length and truncated ORFs, lacking the predicted signal peptides, from both Emoy2 and Maks9 were fused likewise (test constructs). Detached Arabidopsis leaves were cobombarded with a test construct and 35S:GUS. Detection of the product of the avirulence allele of *ATR1Nd* by RPP1-Nd should elicit localized plant cell death (a hypersensitive response) visualized as a reduction in GUS-expressing (blue-stained) plant cells. Reductions in blue-stained cells would not be expected for virulence alleles or for plants that lack *RPP1-Nd*. Cobombardment experiments were performed using a recombinant inbred line (RIL 3860 [3860]) from a genetic cross between Columbia (Col-5) and Nd-1 that lacks *RPP1-Nd* and a transgenic line homozygous for the functional *RPP1-Nd* gene (3860:RPP1Nd) (Figure 3A) (Gordon, 2002). Many blue-stained cells were observed on 3860, regardless of which test construct was cobombarded (Table 2, Figure 3B). Replicate leaves of a single genotype bombarded with the same test construct exhibited substantial variability in their numbers of blue-stained cells. The critical comparison was between pairs of

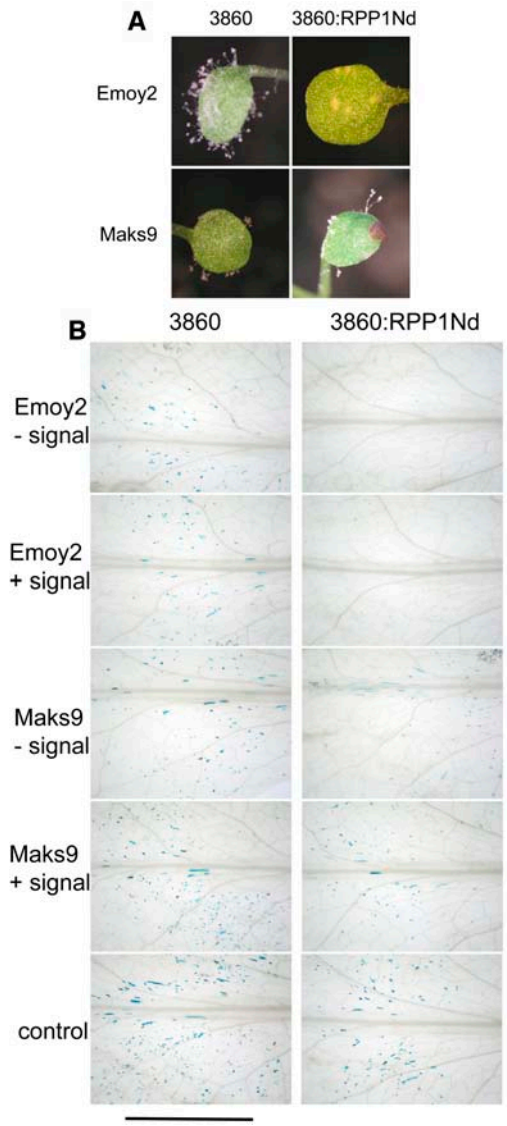

Figure 3. Recognition of ATR1^{NdWsB} by RPP1-Nd.

(A) Pathology of Emoy2 and Maks9 on 3860 (susceptible control) and 3860:RPP1Nd (transgenic) lines.

(B) Emoy2 and Maks9 alleles of *ATR1*^{NdWsB} cloned with (+ signal) or truncated for (− signal) sequences encoding predicted signal peptides and cobombarded with GUS into 3860 and 3860:RPP1Nd lines. Sequences were expressed constitutively from the 35S promoter. Control panels show cobombardment of pK2GW7 (empty vector) with GUS. Bar = 0.5 cm.

leaves (3860 and 3860:RPP1Nd) that were cobombarded together in a single shot. For this reason, the numbers of blue-stained cells in each bombarded leaf in an experiment and the ratio between the numbers of blue-stained cells in pairs of leaves that were shot together are listed in Table 2. The ratios provide a clear measure of the differences between responses to different test constructs, allowing for the between-replicate variability. Cobombardment of 3860:RPP1Nd with Maks9 (virulent) ORFs or a control plasmid produced high numbers of blue-stained plant cells; the mean ratios between the numbers of blue-stained cells in pairs of 3860:RPP1Nd and 3860 leaves ranged from 0.70 to 1.05. By contrast, the Emoy2 (avirulent) ORFs resulted in few or pale blue-stained cells (Table 2, Figure 3B). Therefore, coexpression of the candidate avirulent ORF was sufficient to reduce the expression of GUS specifically in plants carrying the *RPP1-Nd* resistance gene. The reduction in blue-stained cells was more pronounced with the Emoy2 construct lacking the predicted signal peptide (the mean ratio between the numbers of blue-stained cells in 3860:RPP1Nd and 3860 leaves was 0.09) (Table 2, Figure 3B). The full-length Emoy2 construct consistently resulted in numerous blue-stained cells (mean ratio, 0.39; Table 2), although they stained less intensely than the controls (Figure 3B). The detection of ATR1Nd by RPP1-Nd was clearly apparent from macroscopic comparisons between replicate pairs of leaves (Figure 3B) as well as from the quantitative analysis of the relative numbers of blue-stained cells (Table 2). The sequences encoding *ATR1*Nd alleles from

Table 2. Detection of ATR1^{NdWsB} by RPP1-Nd

Test Construct	3860[a]	3860: RPP1Nd[a]	Ratio 3860: RPP1Nd to 3860
Emoy2 − signal	62	16	0.258
	322[b]	32[b]	0.099
	110	5	0.045
	229	10	0.044
	237	1	0.004
			Mean = 0.09
Emoy2 + signal	306	69	0.225
	169	11	0.065
	170	176	1.035
	257[b]	106[b]	0.412
	486	111	0.228
			Mean = 0.39
Maks9 − signal	107	57	0.533
	604	213	0.353
	406[b]	435[b]	1.071
	192	282	1.469
	560	250	0.446
			Mean = 0.77
Maks9 + signal	593[b]	348[b]	0.587
	725	308	0.425
	463	781	1.687
	363	49	0.135
	69	165	2.391
			Mean = 1.05
Control (pK2GW7 empty vector)	276	366	1.326
	73	66	0.904
	68	17	0.250
	56	19	0.339
	707[b]	477[b]	0.675
			Mean = 0.70

[a] The numbers of blue-stained cells present in each pair of leaves (3860 and 3860:RPP1Nd) after cobombardment with 35S:GUS and a test construct and the ratio between control and test leaves within each replicate are shown.

[b] Highlighted leaves were photographed and are shown in Figure 3B.

Noks1, Cala2, Emco5, and Emwa1 were cloned and tested in cobombardment experiments. As predicted, because the isolates are virulent in the presence of *RPP1-Nd*, these alleles did not cause a reduction in blue-stained cells on 3860:RPP1Nd (data not shown).

Recognition of ATR1Nd by RPP1-WsB

The *RPP1* loci from Arabidopsis accessions Nd-1 and Ws-0 determine resistance to different but overlapping sets of *H. parasitica* isolates (Table 1). Whereas a single functional *RPP1* gene in Nd-1 specifies resistance to Emoy2, Hiks1, and Waco5 (Gordon, 2002), three similar genes at the *RPP1* locus in Ws-0 (*RPP1-WsA*, *RPP1-WsB*, and *RPP1-WsC*) specify resistance to Emoy2, Maks9, Noks1, and Cala2 (Botella et al., 1998) (Table 1). Allelism between *RPP1-Nd* and *RPP1-Ws* genes is difficult to determine because of the complexity of *RPP1* loci. Nevertheless, we tested whether *ATR1Nd* alleles are also recognized by *RPP1-Ws* genes. Cobombardment experiments reproducibly indicated that the *ATR1Nd* alleles from Emoy2, Maks9, and Noks1 were recognized by the Ws-0 Arabidopsis accession (few blue-stained cells [data not shown]), whereas the Cala2 allele clearly was not (many blue-stained cells [Figure 4A]). Thus, the alleles were considered good candidates for avirulence determinants recognized by *RPP1-WsB*, because *RPP1-WsB* specifies resistance to Emoy2, Maks9, and Noks1 but not to Cala2 (Botella et al., 1998) (Table 1). Therefore, a homozygous transgenic line carrying *RPP1-WsB* (CW84:RPP1WsB [Botella et al., 1998]) was used in cobombardment experiments using a selection of *ATR1Nd* alleles, and the relative numbers of blue-stained cells in each leaf were scored macroscopically (according to Bryan et al., 2000). The recognition of Emoy2, Maks9, and Noks1 was confirmed as well as the lack of recognition of Cala2 (Table 3, Figure 4B). As predicted, the allele from Emwa1 (virulent on Ws-0) was not recognized (many blue-stained cells [Table 3, Figure 4B]). Because products of specific *ATR1Nd* alleles were recognized by RPP1-WsB, we renamed the gene *ATR1^{NdWsB}*. This notation is necessary because, although RPP1-WsA recognizes an avirulence determinant in Cala2 (Table 1), it does not recognize the Cala2 allele of *ATR1^{NdWsB}* (ATR1^{NdWsB}-Cala2 does not cause cell death in the Ws-0 accession after cobombardment [Figure 4A]).

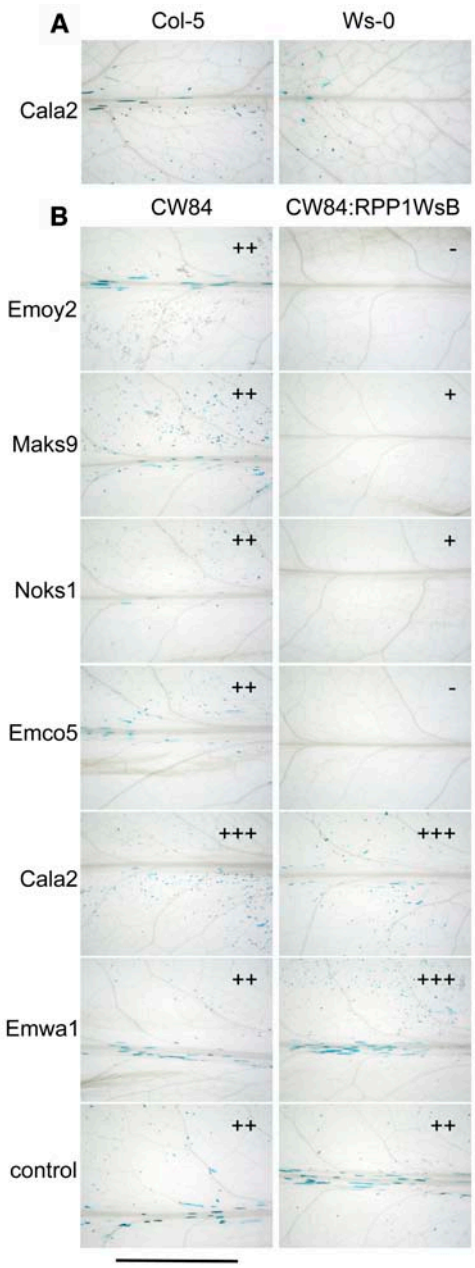

Figure 4. Recognition of ATR1^{NdWsB} by RPP1-WsB.

(A) Cala2 allele, truncated for the predicted signal peptide, cobombarded with GUS into Col-5 (control) and Ws-0 accessions.

(B) Emoy2, Maks9, Noks1, Emco5, Cala2, and Emwa1 sequences, truncated for the predicted signal peptides, cobombarded with GUS into CW84 (susceptible control) and CW84:RPP1WsB (transgenic) lines (Botella et al., 1998). The relative scores (−, +, ++, and +++) of blue-stained cells illustrate and correspond to those shown in Table 3. Sequences were expressed constitutively from the 35S promoter. Control panels show cobombardment of pK2GW7 (empty vector) with GUS. Bar = 0.5 cm.

RPP1-WsB Recognizes an in Planta–Expressed *ATR1^{NdWsB}* Allele from a Virulent Isolate

The Arabidopsis accession Ws-0 is susceptible to the Emco5 isolate, but both the Ws-0 accession (data not shown) and CW84:RPP1WsB (Table 3, Figure 4B) exhibited recognition of ATR1^{NdWsB}-Emco5 in cobombardment experiments. RNA was extracted from plants 24 h after inoculation with different isolates. First-strand cDNA was produced and used as the template for PCR using a selection of primers. PCR amplification using methylenetetrahydrofolate dehydrogenase (MTD; a conserved *H. parasitica* gene from the *ATR1^{NdWsB}* interval) primers demonstrated the presence of *H. parasitica* RNA in each infection after 24 h (Figure 5). Primers corresponding to *ATR1^{NdWsB}*

Table 3. Detection of ATR1^{NdWsB} by RPP1-WsB

Test Construct	CW84[a]	CW84:RPP1WsB[a]
Emoy2	++	−
	++[b]	−[b]
	++	−
	++	+
Maks9	++[b]	+[b]
	++	+
	++	−
	++	−
Noks1	++[b]	+[b]
	+	−
	+	−
	+	+
Emco5	++[b]	−[b]
	++	−
	+	−
	++	+
Cala2	+++[b]	+++[b]
	++	++
	++	+++
	++	+
Emwa1	+++	+
	+++	++
	+	+
	++[b]	+++[b]
Control (pK2GW7 empty vector)	++[b]	++[b]
	+++	++

[a] The relative numbers of blue-stained cells present in CW84 and CW84:RPP1WsB leaves after cobombardment with 35S:GUS and a test construct, truncated for the predicted signal peptide, are shown. The relative scores (−, +, ++, and +++) of blue-stained cells are illustrated in Figure 4B.

[b] Highlighted leaves were photographed and are shown in Figure 4B.

amplified a sequence-verified product from each cDNA preparation, indicating that the gene is expressed in all isolates (Figure 5). PCR amplifications, using an internal *ATR1*^{NdWsB} primer and a primer outside of the *ATR1*^{NdWsB} transcribed region, failed to amplify products, confirming that no contaminating genomic DNA was present (data not shown). Therefore, Emco5 expresses an allele of *ATR1*^{NdWsB} that is recognized by *RPP1-WsB*, yet the isolate is virulent on Ws-0.

The RXLR Motif

The first 67 amino acids of ATR1^{NdWsB} proteins from different *H. parasitica* isolates are relatively conserved compared with the remainder of the proteins (Figure 2). *ATR1*^{NdWsB} lies in a conserved region of synteny with *Avr3a* from *P. infestans*, and AVR3a shows homology with Avr1b-1 from *P. sojae* (Shan et al., 2004; Armstrong et al., 2005). Aligning the amino acid sequences encoded by these three genes revealed no overall sequence conservation (data not shown), but a conserved motif was apparent within 32 amino acids of the predicted signal peptides (Figure 6). The core motif consists of the consensus sequence RXLR followed by a variable length of amino acids comprising >50% acidic amino acids (Asp [D] and Glu [E]) and concluding with Arg (R). As with ATR1^{NdWsB}, most variation between proteins encoded by alleles of *Avr1b-1* occurs beyond the motif (Shan et al., 2004). By searching publicly available sequences of plant pathogenic oomycetes and the sequences of BAC clones spanning the *ATR1*^{NdWsB} interval, we identified eight additional genes from three oomycete species in which a similar motif immediately follows a predicted signal peptide (Figure 6). Among these were secreted Phytophthora proteins (Pieterse et al., 1994; Kamoun et al., 1999; Qutob et al., 2002), predicted Phytophthora extracellular proteins (PEXs) (Torto et al., 2003) from *P. infestans*, and a predicted secreted protein from a *H. parasitica* BAC sequence linked to *ATR1*^{NdWsB} (12I13.1; Figure 6). Alignment of these sequences with the three avirulence proteins further defined the consensus for the motif as RXLR-X$_{5\text{-}21}$-ddEER (uppercase letters denote the consensus amino acid in 10 of 11 sequences; lowercase letters denote the consensus amino acid in more than half of the genes; Figure 6); for simplicity, the term ''RXLR motif'' is used to describe the sequence. The RXLR motif is also found in >40 diverse (in both size and sequence) secreted *P. infestans* proteins (C. Young, T.D. Kanneganti, J. Win, and S. Kamoun, personal communication) and >100 predicted secreted proteins with weak sequence similarity to Avr1b-1 from each of the genome sequences of *P. sojae* and *P. ramorum* (B.M. Tyler, unpublished data). Thus, the RXLR motif is found within different oomycete genomes and within avirulence proteins. The *H. parasitica* ATR13 protein (Allen et al., 2004) represents an alternative form, in which the amino acid residues RXLR are present and preceded by a signal peptide but are not followed by an acidic stretch of amino acids.

Distribution of Sequence Polymorphism in the *ATR1*^{NdWsB} Locus

A sliding-window analysis revealed that the synonymous and nonsynonymous polymorphism is not distributed uniformly across the alleles of *ATR1*^{NdWsB} (Figure 7). Synonymous variation ranges from $\pi = 0.00$ to 0.063, and the central core of the coding region shows no segregating synonymous variation from codon

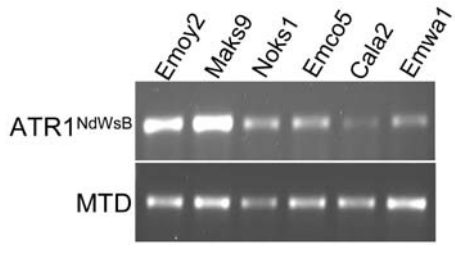

Figure 5. Expression Analysis of ATR1^{NdWsB} in Planta at 24 h after Inoculation.

Sequence-verified PCR products separated on agarose gels and amplified from cDNA derived from Emoy2-, Maks9-, Noks1-, Emco5-, Cala2-, or Emwa1-infected seedlings using ATR1^{NdWsB}-specific primers. Also shown, PCR products amplified from the same cDNA samples using primers corresponding to a control *H. parasitica* gene (MTD).

Figure 6. The RXLR Motif.

(A) Graphic representation of the sequence alignment shown in **(B)**. The height of each amino acid symbol indicates its frequency at that position (Schneider and Stephens, 1990; Crooks et al., 2004). Amino acids Leu (L) and Arg (R) (red) and acidic amino acids (green) are highlighted.
(B) Alignment of the N-terminal regions of predicted protein sequences of ATR1^{NdWsB} from *H. parasitica* (Hp) isolate Emoy2, AVR3a (Armstrong et al., 2005) from *P. infestans* (Pi), Avr1b-1 (Shan et al., 2004) from *P. sojae* (Ps), secreted Pi proteins MY-20-B-07 (Kamoun et al., 1999), IPI-O1, and IPI-O2 (Pieterse et al., 1994) (IPI-O proteins are identical in this region), a predicted Hp secreted protein from BAC sequence 12I13, putative Pi PEXs from BACs 14M19 (GenBank accession number AC146943) and 34A11 (GenBank accession number AC147544), and secreted Ps proteins 3-9f-HA and 1-6b-ZO (Qutob et al., 2002). Dashes indicate gaps in the alignment. Predicted signal peptides (blue), the RXLR motif (red), acidic amino acids (green), and the RGD tripeptide motif (underlined) are highlighted.

positions 81 to 249 (nucleotides 462 to 968; Figure 7). Nonsynonymous variation is lowest in the first one-fifth of the coding region, increases to intermediate levels ($\pi = 0.0451$) in the center of the gene, and is extremely high in the last third of the coding region ($\pi = 0.131$). The region of protein conservation at the 5′ end of the coding region corresponds to the signal peptide and the RXLR motif, described above. The five SNPs differentiating the alleles derived from Emoy2 and Maks9, which were used in the mapping study, are located between nucleotides 495 and 795 (Figures 1 and 7).

Recombination at the *ATR1^{NdWsB}* Locus

Sequence analysis of the *ATR1^{NdWsB}* alleles revealed that recombination had occurred at this locus in the ancestry of these eight *H. parasitica* isolates. The four-gamete test (Hudson and Kaplan, 1985) detected a minimum of two recombination events between nucleotides 591 and 751 and nucleotides 796 and 949. The recombination event between positions 796 and 949 was also detected by an independent analysis using the GENECONV program (www.math.wustl.edu/~sawyer). Further analysis revealed that the Noks1 allele appears to be a chimera of two other alleles in the sample. Specifically, the first 546 nucleotides of the Noks1 allele are 100% identical to the Cala2 allele (Figure 1). From nucleotide 519 (Figure 1) to the end of the sequenced region, the Noks1 allele is 100% identical to the Emco5 allele. Significantly, the Noks1 and Emco5 alleles are recognized by the RPP1-WsB gene, whereas the Cala2 allele is not. This implies that the recognition specificity of alleles of *ATR1^{NdWsB}* by RPP1-WsB is confined to the C-terminal portion of the ATR1^{NdWsB} protein.

DISCUSSION

We have successfully used a map-based cloning strategy to clone the avirulence gene detected by the *RPP1* resistance gene from the Arabidopsis accession Nd-1. Using a cobombardment assay, we found only one allelic form of *ATR1^{NdWsB}* that is recognized by RPP1-Nd. The observation that optimum recognition occurs in the absence of a signal peptide is consistent with cytoplasmic recognition of the avirulence protein. This is

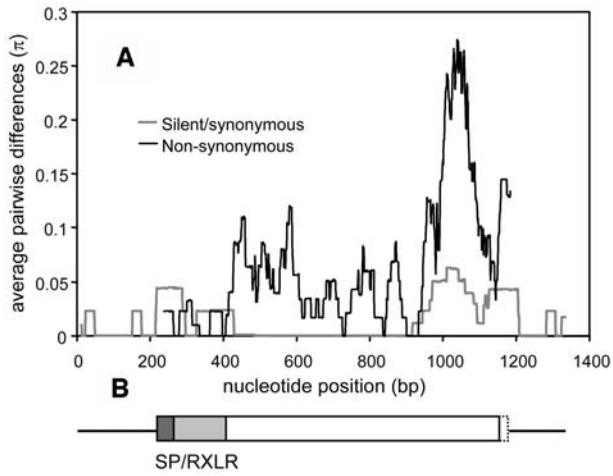

Figure 7. Sliding-Window Analysis.

(A) Average number of pairwise differences between *ATR1^{NdWsB}* alleles from eight *H. parasitica* isolates. Gray lines represent silent and synonymous variations (in noncoding and coding regions, respectively); black lines represent nonsynonymous variations. Values are midpoints of 25-bp windows.
(B) The *ATR1^{NdWsB}* coding region (box) and noncoding regions (solid black lines) aligned with **(A)**. The hatched 3′ end of the coding region represents the slightly longer coding sequences of the Cala2 and Emwa1 alleles. The regions encoding the signal peptide (SP; dark gray) and the RXLR motif (light gray) are indicated.

reminiscent of the weaker recognition of the full-length compared with the truncated avirulence genes *AVR-Pita* from *Magnaporthe grisea* in rice (*Oryza sativa*) (Jia et al., 2000), *AvrL567* from *M. lini* in flax (*Linum usitatissimum*) (Dodds et al., 2004), and *Avr3a* from *P. infestans* in potato (*Solanum tuberosum*) (Armstrong et al., 2005). This suggests that the signal peptide interferes with the recognition of ATR1^{NdWsB}. Perhaps the signal peptide's presence, when expressed inside the plant cell, physically blocks a protein interaction that is required for full recognition; alternatively, the signal peptide may be biologically functional in plant cells, exporting ATR1^{NdWsB} and preventing the accumulation of ATR1^{NdWsB} in the plant cell. By contrast, the *H. parasitica* ATR13 protein was recognized equally efficiently with or without its signal peptide (Allen et al., 2004). This finding suggests that the signal peptide does not physically interfere with ATR13 recognition or that RPP13 is capable of detecting lower levels of ATR13.

In cloning *ATR1*^{NdWsB}, the *H. parasitica* avirulence gene that is perceived by RPP1 from Nd-1, we also identified *ATR1*^{NdWsB} alleles that are perceived by RPP1-WsB from the Ws-0 *RPP1* locus. RPP1-WsB recognizes diverse ATR1^{NdWsB} protein sequences, only one of which was recognized by RPP1-Nd. Surprisingly, although the Ws-0 accession is susceptible when inoculated with the Emco5 isolate, ATR1^{NdWsB}-Emco5 is recognized by RPP1-WsB in our cobombardment assay. Virulent *P. sojae* isolates that carried avirulence alleles of *Avr1b-1* were virulent because they failed to accumulate *Avr1b-1* mRNA (Shan et al., 2004). By contrast, we have demonstrated that *ATR1*^{NdWsB} is expressed by the Emco5 isolate during infection. Conceivably, Emco5 is virulent on Ws-0 because ATR1^{NdWsB} is expressed at low levels, is unstable in vivo, or fails to be taken up by the plant and so remains undetected by RPP1-WsB. An exciting alternative is that the Emco5 isolate somehow evades or suppresses either recognition by RPP1-WsB or the resistance responses triggered by recognition.

ATR1^{NdWsB}-Cala2 is not recognized by RPP1-WsA. Therefore, RPP1-WsA recognizes the product of an avirulence gene other than *ATR1*^{NdWsB} in Cala2, consistent with the hypothesis of Botella and coworkers (1998) that different *RPP1-Ws* genes recognize different avirulence determinants.

ATR1^{NdWsB} differs from other known oomycete or obligate biotroph avirulence genes in both size and the level of encoded protein diversity. ATR1^{NdWsB}, which is >300 amino acids long, is larger than ATR13 (187 amino acids [Allen et al., 2004]), Avr1b-1 (138 amino acids [Shan et al., 2004]), and AvrL567 (150 amino acids [Dodds et al., 2004]). Furthermore, nearly one-third of the coding positions for ATR1^{NdWsB} are polymorphic for two or more amino acid residues. In comparable data sets of allelic variation in avirulence proteins from oomycetes, we observed 10.6% variable amino acid positions among six isolates of *H. parasitica* at ATR13 and 13% variable amino acid positions among six isolates of *P. sojae* at Avr1b-1 (Allen et al., 2004; Shan et al., 2004).

The RXLR Motif

Cloning of *ATR1*^{NdWsB} has broader implications. ATR1^{NdWsB}, avirulence proteins from *P. sojae* and *P. infestans*, and numerous secreted oomycete proteins share a highly conserved motif that we term the RXLR motif. In *ATR1*^{NdWsB}, the RXLR motif is encoded by a region of the gene exhibiting the least nonsynonymous variation. The position of the RXLR residues of the motif in IPI-O proteins is intriguing because it overlaps the RGD tripeptide cell-adhesion motif (Senchou et al., 2004). The RXLR motif is conserved in diverse oomycete genera among dissimilar oomycete proteins, some of which have been characterized as candidate pathogenicity factors (Senchou et al., 2004) or avirulence genes (ATR1^{NdWsB}) (Shan et al., 2004; Armstrong et al., 2005). Conservation of the RXLR motif in such an impressive array of proteins implies a functional significance in oomycetes.

No Cys-rich proteins from oomycetes were found that carry the RXLR motif. Therefore, proteins carrying the RXLR motif differ from the Cys-rich class of pathogen proteins that have been implicated in fungus–plant and oomycete–plant interactions (Templeton et al., 1994).

The RXLR motif shares some notable similarities with the recently described host-targeting signal, which is conserved in numerous, dissimilar proteins from malaria parasites (Plasmodium species) and demonstrated to be required for the translocation of these Plasmodium proteins into the host cell (Hiller et al., 2004; Marti et al., 2004). In particular, the Plasmodium signal is positioned within 60 amino acids of a secretory signal sequence, and the most highly conserved residues of the motif are Arg and Leu residues spaced, as within the RXLR motif, as RXL (Hiller et al., 2004; Marti et al., 2004). ATR1^{NdWsB} and AVR3a (Armstrong et al., 2005) are detected within the cytoplasm of host plant cells; it is tempting to speculate that, like the Plasmodium host-targeting signal (Hiller et al., 2004; Marti et al., 2004), the RXLR motif may play a role in translocating secreted oomycete proteins into the host plant cell. Such a hypothesis is currently untested experimentally.

Relating ATR1^{NdWsB} Sequences to RPP1 Recognition Specificities

The sequences of ATR1^{NdWsB} from eight pathogen isolates can be related to the different recognition specificities of RPP1-Nd and RPP1-WsB. Isolates recognized by RPP1-Nd (Emoy2, Hiks1, and Waco5) share the same allele, but RPP1-WsB–recognized alleles (from Emoy2, Maks9, Noks1, and Emco5) encode four protein sequences with extensive sequence variation. ATR1^{NdWsB}-Maks9, which is not recognized by RPP1-Nd, differs by only five amino acids from ATR1^{NdWsB}-Emoy2, which is recognized by RPP1-Nd. This suggests that the specificity of RPP1-Nd recognition resides within the region spanning these five amino acids (amino acids 92 to 192).

Comparison of the Noks1 and Cala2 alleles revealed that these two sequences are identical for the first 107 codons. Although the RPP1-WsB protein can recognize ATR1^{NdWsB}-Noks1, it cannot recognize the ATR1^{NdWsB}-Cala2 allele. This restricts the region of RPP1-WsB recognition specificity of ATR1^{NdWsB} to the C-terminal portion of the protein, which differentiates the Noks1 and Cala2 alleles from each other. Further comparison of the four proteins encoded by the alleles that are recognized by RPP1-WsB (from isolates Emoy2, Maks9, Noks1, and Emco5) reveals a small region of identical amino acid residues shared by these

four proteins and differentiated from the proteins that are not recognized by RPP1-WsB (Cala2 and Emwa1). If recognition of these four sequences by RPP1-WsB is mechanistically the same (i.e., because of the recognition of a single region within the ATR1^{NdWsB} protein), then the most parsimonious hypothesis is that the region lies between residues 108 and 242. Coincidentally, this region overlaps the region implicated in recognition specificity of ATR1^{NdWsB} by RPP1-Nd. Extremely high levels of nonsynonymous polymorphism relative to synonymous polymorphism were seen at the C terminus of ATR1^{NdWsB}, consistent with this region experiencing diversifying selection. If this region is not involved in *RPP1*-specific recognition, then forces other than RPP1-WsB recognition must be postulated that have resulted in the selective maintenance of such high levels of amino acid diversity. However, it is theoretically possible that RPP1-WsB recognition of the four different ATR1^{NdWsB} proteins is not limited to a single region shared by these four sequences but extends into the highly variable C terminus. Other R-genes with multiple recognition specificities have been described, and in many cases the detected proteins do not show sequence homology (Grant et al., 1995; Rossi et al., 1998; Pedley and Martin, 2003), indicating that the multiple specificity of an R-gene is not necessarily limited to the recognition of a single sequence motif. Additional tests can now be performed, using a range of RPP1 and ATR1^{NdWsB} molecules, to determine which regions of ATR1^{NdWsB} are detected by RPP1 genes.

Dissecting the earliest molecular events that enable a plant to resist infection by downy mildew, or that enable the pathogen to evade detection and establish an infection, requires the study of both plant and downy mildew components involved in the perception of infection. We have cloned a pathogen component, *ATR1^{NdWsB}*, that is under immense diversifying selection pressure but retains a motif, presumably of functional significance, that should assist in identifying new candidate avirulence genes and pathogenicity factors by screening databases of oomycete proteins for its presence. Cloning of *ATR1^{NdWsB}* has revealed that plant RPP proteins encoded by the same genetic locus in different accessions can recognize either single or multiple forms of the same avirulence gene, yet RPP proteins encoded within a single locus of the same accession can recognize different avirulence gene products. Furthermore, virulent isolates can express avirulence alleles of *ATR1^{NdWsB}*. We are now in a position to test the function of the RXLR motif, elucidate the molecular basis of differential RPP recognition capabilities, and investigate what function ATR1^{NdWsB} fulfills during infection.

METHODS

Hyaloperonospora parasitica Isolates

H. parasitica isolates used in this study were gathered from naturally infected *Arabidopsis thaliana* populations; the locations of these populations and cultivation of the isolates were described previously (Holub et al., 1994; Rehmany et al., 2000).

Identification and Subcloning of *ATR1Nd*

H. parasitica BAC clones 9B13, 1G5, and 12I13 (Rehmany et al., 2003) were sequenced by shotgun cloning of sheared BAC fragments, sequencing the subclones, and assembling the sequences (9B13 and 12I13 were sequenced at Lark Technologies, Essex, UK; 1G5 was sequenced at Warwick HRI, Warwick, UK). The sequence prediction software MacMolly Tetra (Softgene, Berlin, Germany) was used to predict protein-coding sequences. Primers 12I13-17029U (5′-CCATTCCATCAAACAACGGCTCTA-3′) and 12I13-18760L (5′-TCTGCGCATAACATTGAAACATCC-3′) were used to amplify PCR products from Emoy2 and Maks9 genomic DNA, extracted as described previously (Rehmany et al., 2000, 2003). SNPs were identified after cycle sequencing (Applied Biosystems, Foster City, CA) using 12I13-18760L. PCR and sequencing were repeated using template DNA from F2 individuals that were previously shown to be recombinant in this region (Rehmany et al., 2003). Primers 12I13-17736U (5′-CCTGACGAGTGCAATGGTAG-3′) and 12I13-19108L (5′-AAGCTCGTTTGAAGACACTGA-3′) were used to amplify the corresponding region from isolates Hiks1, Waco5, Noks1, Cala2, Emco5, and Emwa1. The PCR products were sequenced using primers 12I13-18281U (5′-TCGAACGGGATGATTTGATTGGCG-3′), 12I13-18384L (5′-TGTTACTAGCCTAGTGGCGAGAGC-3′), 12I13-18607U (5′-TCTGAGGACGACGCGAACAACGCC-3′), and 12I13-18760L.

ATR1Nd alleles and the *uidA* gene were cloned into the plant expression vector pK2GW7 (Plant Systems Biology, University of Ghent, Ghent, Belgium) using Gateway recombination (Invitrogen, Carlsbad, CA). Entry clones containing the full-length ORF from Emoy2 and Maks9 were created in pDONR207 (Invitrogen) using primer pairs 12I13-17977UB1 (5′-AAAGCAGGCTTCATGCGCGTCTGCTACTTCGTTCTC-3′) and 12I13-18912LB2 (5′-GAAAGCTGGGTGTTAAACAGAATATATTCTCGAATACTC-3′) and full-length attB1 and attB2 primers (Invitrogen). Entry clones (pDONR207 or pDONR221; Invitrogen) lacking the sequence encoding the signal peptide were similarly produced using primer pairs 12I13-18031UB1 (5′-AAAGCAGGCTCGATGACCGAGTCGTCGGAAACGTCCGGC-3′) and 12I13-18912LB2 for Emoy2 and Maks9, primer pairs 12I13-18031UiB1 (5′-AAAGCAGGCTCGATGACCGAGTCGTCGGAAACGTCCCGC-3′) and 12I13-18912LB2 for Noks1 and Emco5, and primer pairs 12I13-18031UiB1 and 12I13-18912LiB2 (5′-GAAAGCTGGGTGCTACTCGCTCGCGCGCCCTACGAA-3′) for Cala2 and Emwa1. Each pK2GW7 expression clone was sequence-verified before use in the cobombardment experiments.

Transient Expression Assays

The *uidA* gene was cloned into pK2GW7 from the pENTR-GUS (Invitrogen) entry clone for GUS expression in the cobombardment experiments. Cobombardment experiments were performed as described previously (Allen et al., 2004). Briefly, Arabidopsis plants were grown in 10 h of light at 18°C until 7 weeks old. Detached leaves were placed abaxially on a Whatman 3MM paper (Whatman, Middlesex, UK) support on 1% agar plates. DNA (2.5 μg) from each pK2GW7 expression clone was mixed with pK2GW7:GUS (2.5 μg) and loaded onto M17 tungsten particles (Bio-Rad, Hercules, CA); the mixture was used to make four to six replica bombardments using the manufacturer's recommended protocol. For each replicate, a leaf from both test and control plant genotypes were cobombarded together in a single shot. Bombardments were performed using a Bio-Rad PDS-1000 (He) apparatus with 1100-p.s.i. rupture discs. After bombardment, leaves were incubated at 25°C for 20 h. Histochemical GUS staining was performed using 5-bromo-4-chloro-3-indolyl glucuronide (Duchefa Biochemie, Haarlem, The Netherlands) at 37°C for 4 h (Mindrinos et al., 1994), and the tissue was cleared with methanol before scoring the leaves for numbers of blue-stained cells using a light microscope or macroscopically. Experiments were replicated at least twice.

Expression Analysis

RNA was extracted from susceptible plants 24 h after inoculation with *H. parasitica* isolates using the RNeasy plant mini kit (Qiagen, Crawley, UK)

and including a DNase treatment. First-strand cDNA was synthesized using the oligo(dT) CDSIII primer (Invitrogen) and Superscript III reverse transcriptase (Invitrogen). PCR amplifications (37 cycles) were performed using primers 12I13-17977U (5′-ATGCGCGTCTGCTACTTCGTTCTC-3′) and 12I13-18346L (5′-CATGGAGTGGCCCCGGCGCTTCA-3′) or primers MTD5 (5′-GACCCGGCTGCGAAGAAGTATGC-3′) and MTD3 (5′-CCAGCGGCCGACCAACAATG-3′). Products amplified using 12I13-17977U and 12I13-18346L were sequenced using the same primers.

Database Searches for Proteins with a Conserved Motif

All oomycete sequences present in GenBank, as well as the sequences of BACs spanning the *ATR1*Nd locus, were searched for putative proteins fulfilling three criteria: (1) the presence of a signal peptide predicted using SignalP based on previously published criteria (Torto et al., 2003); (2) the occurrence of the sequence RXLR within 40 amino acids of the predicted signal peptide cleavage site; and (3) a stretch of Asp and/or Glu residues in the 30 amino acids after the RXLR motif. The total number of oomycete proteins examined was 1337 (GenBank release November 29, 2004). For genomic sequences, all potential ATG start codons were identified before assessing them for the occurrence of signal peptides or the conserved motif. All analyses were performed using an Apple Macintosh OSX workstation using text string search tools.

Sequence Analysis

The nucleotide sequences from the eight isolates were aligned using ClustalX version 1.8 (Thompson et al., 1997). Minor refinements to this alignment and protein prediction were performed in MacClade version 4.0 (Maddison and Maddison, 2000). Average pairwise differences were calculated and a sliding-window analysis was conducted using DnaSP version 3.51 (Rozas and Rozas, 1999). The numbers of segregating synonymous and nonsynonymous sites and the total numbers of synonymous and nonsynonymous sites were calculated using the SITES program developed by J. Hey (available at http://lifesci.rutgers.edu/~heylab/HeylabSoftware.htm). The χ^2 test was conducted using the online program from GraphPad (http://www.graphpad.com/quickcalcs/chisquared1.cfm). The minimum number of recombination events in the ancestry of these alleles was calculated using DnaSP version 3.51 according to the methods described by Hudson and Kaplan (1985). The GENECONV program (developed by S.A. Sawyer; available at www.math.wustl.edu/~sawyer) was used to determine whether some regions of a pair of sequences had more consecutive identical polymorphic sites than would be expected by chance.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under the following accession numbers: Emoy2 BAC clones 9B13 (AY973543), 1G5 (AY973540), and 12I13 (AY973542 and AY973541); *ATR1*^{NdWsB} alleles from Emoy2 (AY842877), Hiks1 (AY842878), Waco5 (AY842879), Maks9 (AY842880), Emco5 (AY842881), Noks1 (AY842882), Cala2 (AY842883), and Emwa1 (AY842884); and MTD from Emoy2 (AY973539).

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