Molecular Cloning of ATR5^{Emoy2} from *Hyaloperonospora arabidopsidis*, an Avirulence Determinant That Triggers RPP5-Mediated Defense in *Arabidopsis*

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RPP5 is the seminal example of a cytoplasmic NB-LRR receptor-like protein that confers downy mildew resistance in *Arabidopsis thaliana*. In this study, we describe the cloning and molecular characterization of the gene encoding ATR5^{Emoy2}, an avirulence protein from the downy mildew pathogen *Hyaloperonospora arabidopsidis* isolate Emoy2. ATR5^{Emoy2} triggers defense response in host lines expressing the functional RPP5 allele from Landsberg erecta (Ler-0). ATR5^{Emoy2} is embedded in a cluster with two additional ATR5-like (ATR5L) genes, most likely resulting from gene duplications. ATR5L proteins do not trigger RPP5-mediated resistance and the copy number of ATR5L genes varies among *H. arabidopsidis* isolates. ATR5^{Emoy2} and ATR5L proteins contain a signal peptide, canonical EER motif, and an RGD motif. However, they lack the canonical translocation motif RXLR, which characterizes most oomycete effectors identified so far. The signal peptide and the N-terminal regions including the EER motif of ATR5^{Emoy2} are not required to trigger an RPP5-dependent immune response. Bioinformatics screen of *H. arabidopsidis* Emoy2 genome revealed the presence of 173 open reading frames that potentially encode for secreted proteins similar to ATR5^{Emoy2}, in which they share some motifs such as EER but there is no canonical RXLR motif.

Current consensus on plant-pathogen interactions suggests that pathogens use effectors that have been defined as “molecules that alter host-cell structure and function”, which facilitates infection or triggers defense or both (Hogenhout et al. 2009), to adapt to a particular niche and manipulate the host defense network. Numerous effectors from bacteria, parasitic nematodes, oomycetes, and biotrophic fungi have recently been molecularly characterized (Arnold et al. 2009; Elling et al. 2009; Kamoun 2006; Tor 2008). In general, pathogens appear to use effectors to target different components of the immune system, including apoplastic enzymes (Song et al. 2009), cell surface receptors (Meng et al. 2006; Shan et al. 2008), signal transduction cascades (de Torres et al. 2006), transcription machinery (Greenshields and Jones 2008; Kay and Bonas 2009), vesicle trafficking (Schlumberger and Hardt 2006), and protein degradation systems such as the proteasome (Groll et al. 2008).

Pathogens use different mechanisms to deliver effector molecules into a host cell. For instance, many bacterial pathogens use a type III secretion system to deliver effectors directly into the host cytoplasm (Kay and Bonas 2009; Mudgett and Staskawicz 1998). Plant parasitic nematodes typically use a syringe-like stylet to inject their proteins into the plant cell (Elling et al. 2007). Delivery of effectors by biotrophic fungi and oomycetes is poorly understood. However, the process appears to involve intercellular hyphae and haustoria that secrete effectors into the apoplastic space, and the effectors then somehow translocate into the host cells (Catanzariti et al. 2006; Tyler 2009; Whisson et al. 2007).

Cloning fungal and oomycete effectors has been possible using avirulence elicitation as a phenotype for genetic analyses in the host plants that express the corresponding resistance (R) genes. The first examples of avirulence proteins from oomycete species include AvrL567, Avr1b, Avr3a, and ATR13 and ATR1^{NdWsB} from *Melampsora lini*, *Phytophthora sojae*, *P. infestans*, and *H. arabidopsidis*, respectively, (Allen et al. 2004; Armstrong et al. 2005; Dodds et al. 2004; Rehmany et al. 2005; Shan et al. 2004). Sequence conservation (within the N terminal region) among the oomycete avirulence proteins enabled the identification of RXLR-EER motifs, which closely resemble the RXLXE/D/Q motif previously identified in the malaria parasite (*Plasmodium falciparum*) as a domain that is

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Nucleotide sequence data from this article is available in the GenBank and EMBL data libraries under the following accession numbers: FN598669 (ATR5^{Emoy2}), FN598670 (ATR5L1^{Emoy2}), FN598671 (ATR5L2^{Emoy2}), FN598672 (ATR5L^{Noks1}), FN598673 (Deoxyribodipyrimidine photolyase 1, DPL1^{Emoy2}), FN598674 (Deoxyribodipyrimidine photolyase 2, DPL2^{Emoy2}), FN598675 (hypothetical PDZ-domain containing protein, Emoy2), FN598676 (ATR5L1^{Cala2}), FN598677 (ATR5L2^{Cala2}), FN598678 (ATR5L3^{Cala2}), FN598679 (ATR5L4^{Cala2}), and FN598680 (ATR5L5^{Cala2}).

*The e-Xtra logo stands for “electronic extra” and indicates that four supplementary figures and three supplementary tables are published online.

required for translocating the protein into host erythrocytes (Hiller et al. 2004). In addition, Whisson and associates (2007) demonstrated that the β -glucuronidase (GUS) protein could be delivered from *P. infestans* into plant cells when fused with the N-terminal region of Avr3a containing the RXLR and EER motifs. However, delivery did not occur if the RXLR and EER motifs were modified, indicating that these motifs play a significant role in the translocation of oomycete effectors. Recently, Kale and associates (2010) carried out extensive studies on the variation in the RXLR motif and found that the combination of [RKH] x [LMYFI] R[CWPK] would aid the entry of the effector into the plant cell. In addition, they reported that not only the oomycete but also the fungal effectors with variants of RXLR-dEER motif could enter both plant and animal cell by binding to the phospholipid (phosphatidylinositol-3-phosphate, PI3P).

H. arabidopsidis (formerly *Peronospora parasitica*) (Göker et al. 2009) is a natural parasite of *A. thaliana* commonly found in Europe and the genome sequence has recently been published (Baxter et al. 2010). In addition, it has provided an important experimental pathosystem for molecular investigation of *R-Avr* gene pairs. Several *RPP* (resistance to *Peronospora parasitica*) genes, including *RPP1*, *RPP2A*, *RPP2B*, *RPP4*, *RPP5*, *RPP8*, and *RPP13*, have been molecularly characterized and found to encode nucleotide binding-leucine-rich repeat (NB-LRR)-type receptor proteins (Holub 2008), and recent studies with *RPP4* showed that defense genes are under circadian control (Wang et al. 2011). Genetic analyses of avirulence in *H. arabidopsidis* has confirmed an expected gene-for-gene relationship for *ATR* (*A. thaliana* recognized [Holub et al. 1994]) with its corresponding *RPP* gene. *ATR1*^{NdWSB} and *ATR13*Nd have subsequently been cloned and were shown to be members of the RXLR family of effector-like proteins (Allen et al. 2004; Rehmany et al. 2005).

RPP5 was the first downy mildew R protein (a member of the toll interleukin 1 receptor (TIR)-NB-LRR class) to be characterized from *A. thaliana*, isolated from the accession Landsberg *erecta* (Ler-0) and conferring resistance to *H. arabidopsidis* isolates Noco2/Noks1 and Emoy2 (Holub 2007; Parker et al. 1997). Here, we describe the map-based cloning and characterization of the corresponding avirulence gene *ATR5* from the reference genome isolate Hpa-Emoy2. In *ATR5*^{Emoy2}, the canonical translocation signal RXLR motif is not present but the canonical EER motif exists. Sequence analysis indicated variation in gene copy number at the *ATR5* locus among *H. arabidopsidis* isolates.

RESULTS

Mapping *ATR5*.

Table 1 summarizes *RPP* genes that occur in standard *A. thaliana* accessions and the corresponding *ATR* genes in *H.*

Table 1. Downy mildew resistance (*RPP*) genes in *Arabidopsis thaliana* and the corresponding avirulence genes (*ATR*) in *Hyaloperonospora arabidopsidis*^a

<i>Arabidopsis</i> <i>R</i> gene	<i>H. arabidopsidis</i> isolates and their <i>ATR</i> determinants			
	Emoy2	Maks9	Cala2	Noks1
<i>RPP1-Nd</i>	<i>ATR1</i>	V	V	V
<i>RPP2-Col</i>	V	V	<i>ATR2</i>	V
<i>RPP4-Col</i>	<i>ATR4</i>	V	V	V
<i>RPP5-Ler</i>	<i>ATR5</i>	V	V	<i>ATR5</i>
<i>RPP13-Nd</i>	V	<i>ATR13</i>	V	V

^a Genes have been genetically defined for the parent isolates of *H. arabidopsidis* mapping populations described in this publication. *RPP* = resistance to *Peronospora parasitica* (*H. arabidopsidis*), *ATR* = *A. thaliana*-recognized avirulence determinants. V = virulence factors.

arabidopsidis isolates that are relevant to this study. The *RPP5* protein enables recognition of the avirulence determinant designated *ATR5* from Hpa-Emoy2 and Hpa-Noks1 (a single oospore isolate derived from Hpa-Noco2 that retains *ATR5* function). Using an *F*₂ population from a cross between Hpa-Emoy2 and Hpa-Maks9, a single segregating semidominant gene from Hpa-Emoy2 designated *ATR5*^{Emoy2} was characterized (Hpa-Emoy2-avirulent/*F*₁-intermediate/Hpa-Maks9-virulent, 18:47:16, with 2.27 chi square and 0.28 probability [Supplementary Table S1]). We also generated another outcross between isolates Hpa-Cala2 and Hpa-Noks1. Polymerase chain reaction (PCR)-based cleaved amplified polymorphic sequence (CAPS) markers were used to verify recovery of an *F*₁ hybrid isolate designated CaNo *F*₁ (Supplementary Fig. S1). From 72 random CaNo *F*₂ isolates, we observed a single segregating avirulence determinant from Hpa-Noks1, designated *ATR5*^{Noks1} (avirulence/virulence ratio for *ATR5*^{Noks1} was 56:16, with 0.296 chi square and 0.5862 probability, which fits a 3:1 single dominant gene model). Attempts to outcross Hpa-Emoy2 and Hpa-Noks1 for an *F*₂ allelism test were unsuccessful.

We proceeded with the CaNo *F*₂ population to map the *ATR5* locus, using bulked segregant amplification fragment length polymorphism (AFLP) analysis to identify linked molecular markers. The first linked AFLP bands were cloned and converted to CAPS markers and were then used to test individual isolates and establish a mapping interval in a total of 132 CaNo *F*₂ progeny. The reference genome sequence of Hpa-Emoy2 was still being generated during this early *ATR5* mapping work. Therefore, we proceeded with genome walking, using an available Hpa-Emoy2 bacterial artificial chromosome (BAC) library (Rehmany et al. 2003) to screen with the *ATR5*-linked CAPS markers. Identified BAC and BAC end sequences were used to assemble a contig that spans the *ATR5* locus (Supplementary Fig. S2). This region of *H. arabidopsidis* was found to be highly polymorphic and enabled us to rapidly narrow the *ATR5* locus onto a single 127.5 kb BAC (23F10).

At this stage, we used the several CAPS markers to confirm whether *ATR5* from Hpa-Emoy2 and Hpa-Noks1 falls into the same locus. To achieve this, we screened the 80 *F*₂ isolates generated from Hpa-Emoy2 and Hpa-Maks9 outcross with the polymorphic CAPS markers. The markers 22I22_T7 and 13B10_Sp cosegregated with *ATR5*^{Emoy2} phenotypic data, indicating that Hpa-Emoy2 and Hpa-Noks1 share an avirulence determinant that maps to the same locus. The BAC clone spanning the interval was sequenced and was used to identify candidate genes for *ATR5*^{Emoy2} (Fig. 1).

ATR5 locus shows variable gene duplication.

Bioinformatic analysis of the *ATR5* interval in the reference Hpa-Emoy2 genome identified three putative effector-like genes embedded as a cluster. We then focused on this region and rapid amplification of cDNA ends (RACE) PCR was carried out for these and the neighboring genes. Full-length cDNAs for each gene—665, 1,348, 2,004, 1,348, 1,360, and 3,719 bp—were obtained and were compared with the genomic DNA to define exon and intron organization. Genes *B* to *E*, labeled in Figure 1, had a single exon without introns, while the gene *F* had four exons with three introns in the 5' untranslated region. Genes *A* and *C* are duplicates, and BLAST searches indicated that they encode for a putative deoxyribodipyrimidine photolyase (DPL). Gene *A* is a truncated version of gene *C* and both genes were predicted to encode no signal peptide. Genes *B*, *D*, and *E* are also duplicates and are predicted to encode proteins with a signal peptide and an EER motif (Fig. 1), suggesting that these encoded proteins may be secreted and act as putative effectors. Gene *F* encodes a hypothetical protein with multiple PDZ domains (post-synaptic density protein [PSD95], Drosophila disk

large-tumor suppressor [DlgA], and zonula occludens-1 protein [zo-1] [Kim and Sheng 2004]) without a signal peptide. Thus, this combined analysis suggested that genes *B*, *D*, and *E* are the most plausible effector-like candidates for *ATR5* within the mapping interval.

To determine if this gene duplication also occurs in other isolates including Hpa-Cala2 and Hpa-Noks1, we first carried out Southern blot analysis (Fig. 2). A single band was observed in Hpa-Noks1, whereas multiple bands were observed in Hpa-Cala2, indicating the variation in copy number. Subsequent reverse transcription (RT)-PCR studies revealed that Hpa-Cala2 contains five copies, two of which are truncated in the middle of the genes. In contrast, repeated RT-PCR studies (using the primer sequences designed from Hpa-Emoy2) with Hpa-Noks1 detected only one copy of this putative effector.

***ATR5*^{Emoy2} triggers RPP5-dependent cell death.**

Recognition of the *ATR5* product by the RPP5 protein should initiate a localized cell-death response and should, therefore, be amenable for investigation in a cobombardment assay as previously used to characterize *ATR1*^{NdWsB} and *ATR13* (Allen et al. 2004; Rehmany et al. 2005). Detached leaves of *A. thaliana*

accessions CW84 (a broadly *H. arabidopsidis*-susceptible recombinant inbred line generated from a cross between Col-0 and Wassilewskija) and transgenic CW84 containing *RPP5* (Weaver et al. 2006) were cobombarded with the candidate genes (genes *B* to *E*) and the *uidA* gene in a single shot. Experiments were replicated six times. GUS-expressing cells were counted in each experiment (Fig. 3), and the ratio between the numbers of blue-stained cells in leaves that were cobombarded are given in Table 2. The numbers of GUS-expressing cells in CW84 leaves were consistently high regardless of the construct used, indicating the bombardment assay was robust. Although there was variation in the numbers of the GUS-expressing cells among replicates, the ratio between the numbers of blue-stained cells of CW84:*RPP5* and CW84 leaves were consistent. Cobombardment of the CW84:*RPP5* and CW84 leaves with the constructs of genes *B*, *C*, *D*, and *E* or control plasmid gave a mean ratio of 1.070 1.005, 0.175, 0.790, and 1.433 $\pm$ standard error, respectively. These experiments indicated that the expression of gene *D* significantly reduced the number of GUS-expressing cells specifically in CW84:*RPP5*. These findings were verified by repeating the experiments three more times, each with six replicates, and similar results were obtained. Therefore, gene *D* was designated

Fig. 1. Map-based cloning of *ATR5*^{Emoy2}. **A**, Molecular markers used to map *ATR5* from Hpa-Emoy2 and Hpa-Noks1, and the bacterial artificial chromosome (BAC) contig covering the *ATR5* locus (not up to scale). **B**, The BAC that covers the *ATR5*^{Emoy2} region. **C**, The region where *ATR5*^{Emoy2} is embedded. The presence of one truncated and five full open reading frames, designated A through F were determined. Predicted protein sequences of these genes suggest that they encode the following: A, truncated deoxyribodipyrimidine photolyase (DPL); B, a secreted unknown protein; C, deoxyribodipyrimidine photolyase (DPL); D, a secreted unknown protein; E, a secreted unknown protein; and F, a hypothetical protein with multiple PDZ domains. Functional analyses of genes B to F were carried out using cobombardment assays. Only gene D triggered an RPP5-dependent cell death and, thus, was designated *ATR5*^{Emoy2}. Genes B and E have high homology and share the same domain structure with gene D and were designated *ATR5L1*^{Emoy2} and *ATR5L2*^{Emoy2}, respectively. **D**, Domain and motif search in the *ATR5*^{Emoy2} protein identified an initial signal peptide (M1 to R17), an EER (62 to 64 amino acids), and an RGD (97 to 99 amino acids) motif. The position of the canonical RXLR motif is replaced by GRVR in *ATR5*^{Emoy2}.

ATR5^{Emoy2}, while the two paralogs, genes *B* and *E*, were designated *ATR5L1^{Emoy2}* and *ATR5L2^{Emoy2}* (*ATR5L*, *ATR5*-like), respectively. To confirm that the *ATR5*-triggered defense response is solely mediated by *RPP5*, without involving an effect from the genetic background, leaves of Ler-0 that express native *RPP5*, Ler-*rpp5* and Col-0 plants were also bombarded with the test constructs. Results were similar to those obtained with CW84 and CW84:*RPP5*, in that GUS expression was reduced in Ler-0 but not in Ler-*rpp5* and Col-0 (data not shown). To verify our transient expression studies, we placed *ATR5^{Emoy2}*, *ATR5L1^{Emoy2}*, *ATR5L2^{Emoy2}*, and *ATR5L^{Noks1}* under the dexamethasone-inducible promoter and transformed them into Ler-0 plants. Basta-selected transgenic lines (*T*₂s) were treated with dexamethasone, and only the seedlings that carried *ATR5^{Emoy2}* showed chlorosis two days after treatment and died later, confirming the results of our transient studies. Representative lines are shown in Figure 4.

Since Hpa-Noks1 is recognized by *RPP5*, we tested the single copy Hpa-Noks1 allele that we designated *ATR5L^{Noks1}* on CW84 and CW84:*RPP5*. Surprisingly however, no *ATR5L^{Noks1}*-triggered defense response was observed in repeated bombardment experiments (Supplementary Table S2), indicating that there may be another allele that we have not yet detected. Similarly, no *ATR5L^{Noks1}*-triggered defense response was observed in bombarded leaves of Ler-0, Ler-*rpp5*, and Col-0 (data not shown). The three copies of Hpa-Cala2 alleles designated *ATR5L1^{Cala2}*, *ATR5L2^{Cala2}*, and *ATR5L3^{Cala2}* were also tested on CW84 and CW84:*RPP5* plants. No defense response was observed with these alleles (data not shown).

***ATR5^{Emoy2}* restricts**

***Pseudomonas syringae* pv. *tomato* DC3000 growth on *RPP5* carrying *A. thaliana* lines.**

We used the *Pseudomonas syringae* pv. *tomato* DC3000 delivery system (Sohn et al. 2007) to examine the function of

the *ATR5* family of effectors (*ATR5^{Emoy2}*, *ATR5L1^{Emoy2}*, *ATR5L2^{Emoy2}*, and *ATR5L^{Noks1}*) in CW84 and CW84:*RPP5* lines. First, we tested whether these effectors would trigger an *RPP5*-mediated hypersensitive response (HR) elicitation using this delivery system. No visible HR symptoms developed at 24 h postinoculation in the leaves of CW84 and CW84:*RPP5* infiltrated with DC3000 carrying empty vector or pEDV6:*ATR5*. We also checked whether these effectors would restrict or enhance the growth of DC3000. Both CW84 and CW84:*RPP5* allowed full growth of DC3000 delivering either *ATR5L1^{Emoy2}* or *ATR5L^{Noks1}*. However, delivery of *ATR5^{Emoy2}* reduced the growth of DC3000 in CW84:*RPP5* at a value of 1 log (Fig. 5), indicating that the recognition of *ATR5^{Emoy2}* by *RPP5* in *A. thaliana* caused restricted in planta bacterial growth when delivered by DC3000. In addition, no enhanced bacterial growth was observed with DC3000 carrying *ATR5^{Emoy2}*, *ATR5L1^{Emoy2}*, *ATR5L2^{Emoy2}*, and *ATR5L^{Noks1}*.

The N-terminal region of *ATR5^{Emoy2}* is not essential for detection by *RPP5*.

We generated test constructs from *ATR5^{Emoy2}* that lacked either the region encoding for the signal peptide or the region encoding 66 amino acids (aa) including the EER motif at the N-terminal of the protein. These constructs were then cobombarded with *uidA* gene into the leaves of CW84 and CW84:*RPP5*. Full-length *ATR5^{Emoy2}* and the pEarlyGate100 vector were also included as controls. A high number of GUS-

Fig. 2. Southern analysis of *ATR5* locus. Genomic DNA was isolated from Ws-*eds1* plants infected with Hpa-Cala2, Hpa-Emoy2, and Hpa-Noks1 and was digested with *EcoRI* (E), *HindIII* (H), and *PstI* (P). DNA from uninfected Ws-*eds1* plants was digested with *EcoRI* only and was used as a control. Membrane was hybridized with ³²P-labeled *ATR5^{Emoy2}* DNA. Only one copy of *ATR5^{Emoy2}* was found in the isolate Hpa-Noks1, whereas Hpa-Emoy2 and Hpa-Cala2 isolates had multiple copies.

Fig. 3. Recognition of *ATR5^{Emoy2}* by *RPP5*. Detached leaves of *Arabidopsis* accessions CW84 and CW84:*RPP5* were cobombarded with test constructs and the *uidA* gene in a single shot. Cobombardment of empty vector (pEarlyGate100) and the *uidA* gene served as a control. The number of β-glucuronidase (GUS)-expressing cells was counted in each experiment, and the mean ratios of GUS-expressing cells between pairs of CW84 and CW84:*RPP5* leaves were determined. The number of GUS-expressing cells was significantly reduced in CW84:*RPP5* when bombarded by the construct containing *ATR5^{Emoy2}*.

expressing cells was obtained with all constructs in the leaves of CW84 (Fig. 6). In contrast, a high number of GUS-expressing cell was observed only in the leaves of CW84:RPP5 bombarded with the control vector (Table 3). However, the ratio between the numbers of GUS-expressing cells in pairs of CW84 and CW84:RPP5 were consistently low with the full-length and deleted *ATR5^{Emoy2}* test constructs, indicating that the N-terminal region of the *ATR5^{Emoy2}* protein is not required for the avirulence function, and the *ATR5^{Emoy2}*-RPP5 interaction occurs within the cytoplasm.

Sequence and expression analysis of *ATR5^{Emoy2}*.

BLAST searches against the *H. arabidopsidis* Unigenes database demonstrated *ATR5^{Emoy2}*, *ATR5L1^{Emoy2}*, and *ATR5L2^{Emoy2}* were all expressed. We used primers specific to *ATR5^{Emoy2}* and confirmed the expression in spores as well as during infection, by RT-PCR (Fig. 7).

Domain and motif searches of the *ATR5^{Emoy2}* protein identified a signal peptide (M1-R17), an EER (62 to 64 aa), and an RGD (97 to 99 aa) motif (Fig. 8). Hydropathy plot analysis indicated that *ATR5^{Emoy2}* is a soluble protein with no obvious transmembrane domain. Despite the high amino-acid sequence identity to *ATR5L1^{Emoy2}* and *ATR5L2^{Emoy2}*, the triggering of the defense response only by *ATR5^{Emoy2}* suggests that substitutions of certain amino acids in the protein abolish its recog-

nition by RPP5. Comparison of the amino acid sequences of *ATR5^{Emoy2}* with *ATR5L1^{Emoy2}*, *ATR5L2^{Emoy2}*, *ATR5L^{Noks1}*, *ATR5L1^{Cala2}*, *ATR5L2^{Cala2}*, and *ATR5L3^{Cala2}* showed that these proteins are identical at the N-terminus up to F77 and polymorphism increased towards the C-terminus (Fig. 8).

Phylogenetic analysis demonstrates the close homology between these proteins (Fig. 9), with 81% similarity separating the most distantly related proteins, *ATR5^{Emoy2}* and *ATR5L2^{Cala2}*. *ATR5L1^{Cala2}*, *ATR5L3^{Cala2}*, *ATR5L1^{Emoy2}*, *ATR5L2^{Emoy2}*, and *ATR5L^{Noks1}* form a closely related clade, with *ATR5^{Emoy2}* and *ATR5L2^{Cala2}* being more distantly related to the other proteins. Alignment of nucleotide sequences of *ATR5^{Emoy2}* paralogs and homologs from Hpa-Emoy2, Hpa-Cala2, and Hpa-Noks1 indicates the level of polymorphism (Supplementary Fig. S3). Comparisons of Hpa-Emoy2 copies alone revealed 65 nt to be polymorphic, 62 of them corresponding to a single nucleotide polymorphism and three corresponding to insertions or deletions.

H. arabidopsidis has a repertoire of *ATR5^{Emoy2}*-like effectors.

As stated, we found that *ATR5^{Emoy2}* has no canonical RXLR motif, and yet, it triggers a defense response. Thus, we com-

Table 2. Detection of *ATR5^{Emoy2}* by RPP5

Construct ^a	CW84 ^b	CW84:RPP5	Ratio ^c	Mean
<i>ATR5^{Emoy2}</i>	607	42	0.069	0.175 ^d
	112	17	0.152	
	304	136	0.447	
	698	109	0.156	
	534	95	0.178	
	444	20	0.045	
<i>ATR5L1^{Emoy2}</i>	608	866	1.424	1.070 ^e
	416	747	1.796	
	960	492	0.513	
	633	634	1.002	
	406	287	0.707	
	612	599	0.979	
<i>ATR5L2^{Emoy2}</i>	937	1,472	1.571	0.790 ^e
	1,237	221	0.179	
	1,055	704	0.667	
	566	313	0.553	
	490	386	0.788	
	653	640	0.980	
DPL ^{Emoy2f}	707	583	0.825	1.005
	765	803	1.050	
	731	1,254	1.715	
	860	982	1.142	
	1,395	1,211	0.868	
	947	408	0.431	
Control ^g	627	584	0.931	1.433
	526	527	1.002	
	358	701	1.958	
	272	835	3.070	
	706	975	1.381	
	1,288	328	0.255	

^a Test constructs were generated by cloning genes *B*, *C*, *D*, and *E* in the interval under the 35S promoter in pEarlyGate100 vector.

^b The numbers represent the blue-stained cells in leaves of CW84 and CW84:RPP5, after cobombardment with 35S:GUS.

^c The ratio of CW84:RPP5 to CW84.

^d The gene *D* construct gave the lowest number of blue-stained cells and, thus, is designated *ATR5^{Emoy2}*.

^e Genes *B* and *E* were highly similar in their predicted amino-acid sequence and, thus, were designated as *ATR5L1^{Emoy2}* and *ATR5L2^{Emoy2}*, respectively.

^f DPL = deoxyribodipyrimidine photolyase, corresponds to gene *C* in the interval.

^g pEarlyGate100 empty vector.

Fig. 4. Activation of *ATR5^{Emoy2}* induces cell death in a Ler-0 background. *ATR5^{Emoy2}*, *ATR5L1^{Emoy2}*, *ATR5L2^{Emoy2}*, and *ATR5L^{Noks1}* under the control of a dexamethasone (Dex)-activated promoter were transformed into Ler-0 plants, and segregating T₂ lines were treated first with Basta and then with 30 μ M Dex. Lines that carry *ATR5^{Emoy2}* became chlorotic 2 days after treatment and later died. **A**, Nontransgenic Ler-0, **B**, nontransgenic Ler-0, **C**, Ler-0 transgenic with *ATR5L1^{Emoy2}*, **D**, Ler-0 transgenic with *ATR5L2^{Emoy2}*, **E**, Ler-0 transgenic with *ATR5^{Emoy2}*, and **F**, Ler-0 transgenic with *ATR5L^{Noks1}*.

pared ATR5^{Emoy2} against the published RXLR-type of effectors from *H. arabidopsidis* (Win et al. 2007), using BLAST searches to determine if ATR5^{Emoy2} would show homology to any members of this family. Interestingly, ATR5^{Emoy2} shared 24.5% aa identity with the Hpa_Contig214.3_F41. The homology was mainly at the N-terminus, but with the amino-acid sequence GRVR replacing the RXLR motif in ATR5^{Emoy2} (Supplementary Fig. S4).

The question then arises as to whether there are any other potential effectors in *H. arabidopsidis* without a canonical RXLR motif, as observed with ATR5^{Emoy2}. To determine this, we searched the reference genome for open reading frames with an N-terminal signal peptide. From 7,740 putative secreted proteins, 201 have an EER (but no canonical RXLR) motif within the first 100 aa of the mature protein. Proteins with predicted transmembrane helices were eliminated, leaving 173 candidates in addition to ATR5^{Emoy2} and its paralogs ATR5L1^{Emoy2} and ATR5L2^{Emoy2}. Their amino-acid sequences, signal peptide scores (Signal P v3, NN score, and hidden Markov model), possible cleavage, and EER positions are given in Supplementary Table S3. Interestingly, in addition to ATR5^{Emoy2}, ATR5L1^{Emoy2}, and ATR5L2^{Emoy2}, only three other members carry the RGD motif.

DISCUSSION

We used a conventional map-based cloning approach to determine the molecular identity of ATR5^{Emoy2}. Here, we present evidence that ATR5^{Emoy2} i) encodes a putative effector without a canonical RXLR motif, ii) displays copy-number variation among different isolates of *H. arabidopsidis*, and iii) triggers an RPP5-dependent cell death. We also determined that *H. arabidopsidis* possesses a large group of potential effectors with similar structure to ATR5^{Emoy2}. The isolation of ATR5^{Emoy2}, together with the cloning of RPP5 a decade ago (Parker et al. 1997), provides the third molecularly characterized ATR/RPP gene pair from the *A. thaliana*–*H. arabidopsidis* pathosystem.

Mapping of the ATR5 locus to a single BAC using 192 F₂ progeny indicates a high degree of recombination in this chro-

mosomal region of *H. arabidopsidis*. Confirmation of the ATR5 locus in two different crosses illustrates the robustness of the genetic evidence and, also, the polymorphic nature of the region among standard isolates of *H. arabidopsidis*. Single base-pair changes, small insertions or deletions, gene duplica-

Fig. 6. Recognition of truncated ATR5^{Emoy2} by RPP5. Detached leaves of *Arabidopsis* accessions CW84 and CW84:RPP5 were cobombarded with test constructs i) ATR5^{Emoy2} full coding region, ii) ATR5^{Emoy2} without signal peptide, and iii) ATR5^{Emoy2} without the N-terminal region including the EER motif and the *uidA* gene in a single shot. Empty vector (pEarleyGate100) and the *uidA* gene were cobombarded to serve as control. The number of β -glucuronidase-expressing cells was counted in each experiment and the mean ratio in pairs of CW84 and CW84:RPP5 leaves were determined.

Fig. 5. In planta bacterial growth (*Pseudomonas syringae* pv. *tomato* DC3000) delivering ATR5^{Emoy2} and its homologs. Leaves of six-week-old *Arabidopsis* accessions CW84 and CW84:RPP5 were hand-inoculated using a blunt-ended 1-ml syringe with DC3000 strains carrying pEDV or pEDV:ATR5 (10⁶ CFU/ml). Each bar represents the mean number of bacterial colonies recovered on selective agar medium containing appropriate antibiotics from four independent replicates. Bacterial growth was determined by counting colonies recovered from 0 and 3 days after infiltrations. An asterisk represents significant difference ($P < 0.05$) in bacterial growth compared with that obtained from CW84 3 days postinoculation (dpi).

tion, and deletion of large DNA fragments contribute significantly to the evolution of a genome (Ohno 1970). Segmental duplications (segments of DNA with nearly identical sequences) have been reported to be hot spots for nonallelic homologous recombination and the sites for genetic mutations, including duplications, deletions, and inversions leading to genomic instability (Jiang et al. 2007). This appears to be the case for *ATR5*, as analysis of the locus in the isolates Hpa-Emoy2, Hpa-Cala2, and Hpa-Noks1, using bioinformatics, comparative genomics, and targeted experiments such as RT-PCR analysis, revealed that a segment of DNA (containing a photolyase and a copy of *ATR5^{Emoy2}*) occurs twice in the Hpa-Emoy2 genome. Although sequence information of the *ATR5* locus was not obtained from Hpa-Cala2, RT-PCR analysis showed two truncated and three full copies of *ATR5^{Emoy2}*, indicating the possibility of a similar segmental duplication at this region in this isolate.

Gene clustering and a variation in the copy number of effectors is a common feature in oomycete pathogens (Haas et al. 2009). For example, *Avr1a* and *Avr3a* of *Phytophthora sojae* encode RXLR-type effectors and both show copy-number variation in different strains (Qutob et al. 2009). Similarly, genes that encode necrosis-inducing proteins in *P. sojae* and *ipiO* and *ipiB* in *P. infestans* are also clustered in the genome (Pieterse et al. 1994; Qutob et al. 2002). At the *ATR5* locus, not only the effector-like genes show variation in copy number but also the other noneffector like genes such as Gene A and C, which encode DPL.

Since *ATR5^{Emoy2}* triggers a significant RPP5-dependent defense response in *RPP5*-carrying *A. thaliana* lines, it is tempting to speculate that the *ATR5* locus in Hpa-Emoy2 has evolved to avoid host recognition while retaining effector function, which might be necessary to maintain fitness of the pathogen. We cannot exclude the possibility that the other paralogs,

ATR5L1^{Emoy2} and *ATR5L2^{Emoy2}*, as well as alleles such as *ATR5L1^{Cala2}*, *ATR5L2^{Cala2}* and *ATR5L3^{Cala2}* could also be avirulence determinants that are recognized instead by *R* genes other than *RPP5* or *RPP5* variants. This would require screening of natural variation in *A. thaliana* to identify the alternative *R* genes. Such a study has been carried out with *ATR13* and at least one gene, unlinked to *RPP13* in *A. thaliana*, has been shown to detect a different subgroup of *ATR13* alleles (Hall et al. 2009).

The *RPP5* locus in *A. thaliana* Ler-0 corresponds with a cluster of seven paralogs of TIR-NB-LRR encoding genes in Col-0, including *RPP4* and *SNC1* (all members of the TNL-E clade) (Meyers et al. 2003; Noel et al. 1999). Since Hpa-Emoy2 carries an *ATR4* allele, the question then arises as to whether one of the *ATR5* paralogs in Hpa-Emoy2 is recognized by *RPP4*. Genetic evidence indicates that *ATR4* and *ATR5* are unlinked. In addition, cobombardment of *ATR5^{Emoy2}* paralogs with the *uidA* gene into Col-0 plants did not trigger any defense response, indicating that none of these genes are recognized by an *R* gene in Col-0.

The lack of an avirulence phenotype with the *ATR5L^{Noks1}* allele is intriguing because the corresponding *H. arabidopsidis* isolate Hpa-Noks1 triggers a resistant response on Ler-0 and CW84:RPP5 but not on Ler-rpp5, and we mapped the *ATR5* locus using the progeny of a cross between Cala2 and Noks1. This result was confirmed by several replicated and repeated experiments. Explanations to consider include one or both the following possibilities: i) *ATR5L^{Noks1}* may be interacting with another effector protein when delivered naturally into the host cell by the pathogen, and the RPP5 protein might enable detection of the two-effector combination; and ii) the functional characterization experiments used in this study require expression in a non-*H. arabidopsidis* background (*Arabidopsis* or *Pseudomonas*) and possible post-translational modifications may not occur as they would in *H. arabidopsidis*. Thus, *ATR5L^{Noks1}* may go through some post-translational modifications, which alter the end product that can elicit RPP5-mediated cell death. The genetic analysis clearly suggests that *ATR5L^{Noks1}* segregates as a single locus in Hpa-Noks1. It should be noted that the transient expression studies with these putative effectors were carried out with a clone using bombardment assays rather than being delivered by another isolate of *H. arabidopsidis*.

Table 3. Detection of different versions of *ATR5^{Emoy2}* by RPP5

<i>ATR5^{Emoy2}</i>				
Constructs	CW84	CW84:RPP5	Ratio ^a	Mean
Full	125	5	0.040	0.036
	756	28	0.037	
	618	71	0.115	
	193	0	0.000	
	721	6	0.008	
	489	9	0.018	
	475	1	0.002	
Δ SP ^b	327	1	0.003	0.045
	1,086	2	0.002	
	233	0	0.000	
	158	17	0.108	
	242	38	0.157	
	228	16	0.070	
	435	6	0.014	
Δ EER ^c	160	2	0.013	0.063
	376	1	0.003	
	661	179	0.271	
	752	5	0.007	
	418	688	1.646	
	790	8	0.010	
	603	367	0.609	
Control ^d	548	495	0.903	2.188
	258	248	0.961	
	13	117	9.000	

^a The ratio of CW84:RPP5 to CW84.

^b In this construct, the signal peptide region was removed from the coding region of the *ATR5^{Emoy2}* gene.

^c In this construct, the N-terminal region encoding for 66 amino acids including the EER motif has been removed from the coding region of the *ATR5^{Emoy2}* gene.

^d pEarleyGate100 empty vector.

Fig. 7. Expression analysis of *ATR5^{Emoy2}*. Total RNA was isolated from uninfected Ws-eds1 plants or plants infected with Hpa-Emoy2 at 1, 2, 3, and 7 days after inoculation. Reverse transcription-polymerase chain reaction (RT-PCR) analyses were performed with *ATR5* gene-specific primers, using 0.5 μ g of total RNA as a template. The PCR products were separated on a 1.5% agarose gel and were stained with ethidium bromide. The products were of the expected size and their sequences were identical to that of *ATR5^{Emoy2}*. *Hpa-actin* gene was used as a control in RT-PCR assays. *ATR5^{Emoy2}* is expressed in spores as well as during infection.

Although *ATR5^{Emoy2}* and *ATR5L^{Noks1}* share 86.6% amino-acid similarity, there is sufficient sequence difference to influence the stability or function of the protein within the cell. Post-translational modifications (PTM) of proteins include phosphorylation, SUMOylation, methylation, ubiquitination, and acetylation among many others, and each PTM leads to a distinct functional outcome (Lonard and O'Malley 2007). Analyses of *ATR5^{Emoy2}* and *ATR5L^{Noks1}* for post-translational modifications, using several bioinformatic tools, suggest the presence of different glycosylation and phosphorylation sites and numbers. It is therefore possible that *ATR5L^{Noks1}* requires the aid of PTM by the pathogen before being delivered to the plant cell to activate *RPP5*-mediated resistance. It would be ideal to deliver *ATR5L^{Noks1}* through a Ler-0 virulent isolate of *H. arabidopsidis* such as Cala2 to determine if the PTM plays a role. However, genetic transformation of *H. arabidopsidis* has not yet been established.

To date, several fungal- or oomycete-secreted proteins have been shown to be taken up by plant cells. In this study, using bombardment and *Pseudomonas syringae* pv. *tomato* DC3000 delivery systems, we showed that the recognition of *ATR5^{Emoy2}*

by *RPP5* may be indirect and takes place within the cytoplasm, which suggests that *ATR5^{Emoy2}* somehow enters the plant cell. Domain and motif searches revealed that the *ATR5^{Emoy2}* has a signal peptide, a dEER, and an RGD motif. RXLR or RXLR-dEER motifs have been shown to act as translocation signals in some of the oomycete effectors (Whisson et al. 2007). *ATR5^{Emoy2}* has no canonical RXLR motif, and comparison with the RXLR type effectors indicate that this motif is replaced by GRVR. It would be interesting to determine whether the GRVR has a similar function to RXLR and whether the dEER motif is sufficient for its own translocation.

The RGD tripeptide has been described as a motif in other oomycete effectors, including IPI-O and IPI-B of *P. infestans* (Pieterse et al. 1994). This motif is involved in the disruption of adhesion between the cell wall and the plasma membrane (Senchou et al. 2004). Interestingly, *A. thaliana* lectin-RLK type receptors have been shown to recognize RGD-containing effectors such as IPI-O of *P. infestans* (Gouget et al. 2006). Similarly, ToxA (Sarma et al. 2005), a small host-selective toxin secreted by fungal pathogen *Pyrenophora tritici-repentis*, contains an RGD motif and is, consequently, internalized

<i>ATR5^{Emoy2}</i>	MRLISPALVSTAIQA RHVNSSAPVDSAMTEANPLASAHPPDVGVDGVPAG GRVRNP DP TEERT	65
<i>ATR5L1^{Emoy2}</i>		65
<i>ATR5L2^{Emoy2}</i>		65
<i>ATR5L1^{Cala2}</i>		65
<i>ATR5L2^{Cala2}</i>		65
<i>ATR5L3^{Cala2}</i>		65
<i>ATR5L^{Noks1}</i>		65
<i>ATR5^{Emoy2}</i>	PGESFMEA INFKIFKL VQEAQGR ILGLPEQ PRGD MEWLE RYGQDA ILHYLETGDKDPSQLEKKYD	130
<i>ATR5L1^{Emoy2}</i>	D.....D.....E.....	130
<i>ATR5L2^{Emoy2}</i>	D.....D.....	130
<i>ATR5L1^{Cala2}</i>	E.....D.....E.....	130
<i>ATR5L2^{Cala2}</i>	E.....D.....K.....	130
<i>ATR5L3^{Cala2}</i>	E.....D.....K.....	130
<i>ATR5L^{Noks1}</i>	D.....D.....E.....	130
<i>ATR5^{Emoy2}</i>	QLLDELKNAPNLEVE ILES HALFLAYMEEVAKPAVQTT PKLNEQPDKFAWAMIN KARRNAKPGI	195
<i>ATR5L1^{Emoy2}</i>	D.....D.....G.....R.....D.....	195
<i>ATR5L2^{Emoy2}</i>	Y.....D.....G.....N.....D.....W.....E.....	195
<i>ATR5L1^{Cala2}</i>	D.....G.....R.....K.....D.....S.....	195
<i>ATR5L2^{Cala2}</i>	D.....G.....R.....K.....D.....D.....W.....E.....R.....	195
<i>ATR5L3^{Cala2}</i>	D.....G.....R.....K.....D.....D.....S.....	195
<i>ATR5L^{Noks1}</i>	Y.....D.....G.....R.....N.....D.....W.....	195
<i>ATR5^{Emoy2}</i>	RNPYKSLNI PLVENYIKKYNA-----FIELRQRELTLLDTFS CAFNHNTVKLAKFLAMVDT FSPK	255
<i>ATR5L1^{Emoy2}</i>	Q.....-.....-.....E.....T.....LGN.....KH	255
<i>ATR5L2^{Emoy2}</i>	FE.....NCRKVL.....R.....T.....LN.....EH	260
<i>ATR5L1^{Cala2}</i>	NCRKVL.....H.....R.....E.....T.....LGN.....KH	260
<i>ATR5L2^{Cala2}</i>	HWKCVL.....GR.....Q.....R.....E.....LN.....PQN	260
<i>ATR5L3^{Cala2}</i>	NCRKVL.....H.....R.....E.....T.....LGN.....KH	260
<i>ATR5L^{Noks1}</i>	FE.....NCRKVL.....R.....T.....L.....EH	260
<i>ATR5^{Emoy2}</i>	RTFVLAMRI ELSEIWI EEKRTIAEVASI LGISTITGYAKNRLSAGTFVRFI YQLAKTNEQLGPD I	320
<i>ATR5L1^{Emoy2}</i>	M.....K.....SE.....E.....D.....FR.....L.....H.....F.....K.....	320
<i>ATR5L2^{Emoy2}</i>	SR.....E.....D.....FR.....L.....H.....F.....K.....	325
<i>ATR5L1^{Cala2}</i>	M.....SE.....E.....D.....FR.....L.....H.....F.....K.....	325
<i>ATR5L2^{Cala2}</i>	EK.....R.....T.....TK.....K.....K.....TP.....F.....KM.....M.....DR.....L.....K.....K.....V.....	325
<i>ATR5L3^{Cala2}</i>	M.....SE.....E.....D.....FR.....L.....H.....F.....K.....	325
<i>ATR5L^{Noks1}</i>	SR.....E.....D.....FR.....L.....H.....F.....K.....	325
<i>ATR5^{Emoy2}</i>	VKDLVKTFGPDRTTELLTRMKTVSPRMFTILKDHMDVRLKETGVTPN*	368
<i>ATR5L1^{Emoy2}</i>	N.....NIL.....A.....R.....F.....EK.....NGN.....RHS	367
<i>ATR5L2^{Emoy2}</i>	N.....SIL.....A.....R.....I.....NS.....I.....Y.....NGN.....RHS	372
<i>ATR5L1^{Cala2}</i>	N.....NIL.....A.....R.....P.....KK.....Y.....N.....NGN.....RHS	371
<i>ATR5L2^{Cala2}</i>	N.....NI.....LA.....R.....I.....K.....KS.....KK.....Y.....N.....NGN.....RHS	371
<i>ATR5L3^{Cala2}</i>	N.....NIL.....A.....R.....P.....KK.....Y.....N.....NGN.....RHS	371
<i>ATR5L^{Noks1}</i>	N.....SIL.....A.....R.....NS.....I.....Y.....NGN.....RHS	372

Fig. 8. Alignment of amino-acid sequences of *ATR5^{Emoy2}* paralogs and homologs from Hpa-Emoy2, Hpa-Cala2 and Hpa-Noks1. Amino-acid sequences were predicted from cDNAs isolated from each isolate and were aligned using CLC Combined Workbench (Version 3.6, CLC Bio, Aarhus, Denmark). Dots indicate amino acids identical to *ATR5^{Emoy2}* sequences, dashes indicate gaps in the alignment. The predicted N-terminal signal peptide, the dEER motif, and the RGD peptides are boxed in the *ATR5^{Emoy2}* sequence. The GRVR motif is underlined to show that there is no canonical RXLR motif. Numbers at the site correspond to the amino-acid position of each protein.

by wheat mesophyll cells (Manning et al. 2008). Similarly, in animal cells, the integrin family of cell adhesion receptors recognize the RGD motifs, and the residues surrounding the RGD motif provide specificity as well as high affinity for each integrin-ligand pair (Takagi 2004).

Although some effectors may not have the RXLR motifs, different combinations of amino acids in the RxLR region could also act as a translocation signal. Extensive studies of this motif revealed that the combinations of [RKH] x [LMYFI] R{CWPK} would aid the entry of the effector into the plant cell (Kale et al. 2010). The ATR5^{Emoy2} has the KIFK and RIL[G/D] amino acids at the N-terminal between the conserved dEER and the RGD motifs, which would fit this description and may act as a translocation signal, but this needs to be experimentally confirmed. Considering the role of RGD and dEER motifs in the translocation or internalization of proteins and the possible role of the encrypted translocation signals, such as KIFK and RIL[G/D], it is plausible that ATR5^{Emoy2} may enter the cell through these motifs at the N-terminal. Once inside, only the interaction of ATR5^{Emoy2} with host proteins may be detected by RPP5, leading to an immune response, while the paralogs of ATR5^{Emoy2} remains undetected.

Although it is generally assumed that effector proteins are somehow involved in suppressing defense responses, our experiments using delivery by *Pseudomonas syringae* pv. *tomato* DC3000 did not provide evidence for enhancement in bacterial growth, as was previously observed with ATR13 and ATR1 (Sohn et al. 2007). BLAST searches with ATR5^{Emoy2} provided no suggestions for the function of this effector. Solving the molecular structure and determining the interactors will help to uncover the virulence function of ATR5^{Emoy2}. Understanding how these proteins contribute to the pathogen's fitness is likely to provide novel insights into the biotrophic interactions between the oomycete pathogens and their host plant.

MATERIALS AND METHODS

Plant lines.

A. thaliana accessions Columbia (Col-0, Col-5) and Landsberg *erecta* (Ler-0) were obtained from the European Arabidopsis Stock Centre. Wassilewskija-eds1.1 (Ws-eds1) (Parker et al. 1997), Ler-rpp5 (Muskett et al. 2002), and CW84, CW84:RPP5, and CW84:RPP4 (Weaver et al. 2006) were obtained from J. Jones (Sainsbury Laboratory, Norwich, U.K.).

Downy mildew isolates and pathology methods.

All *H. arabidopsidis* isolates collected from natural infection and the inoculation method used in this study have been described elsewhere (Tör et al. 2002) and all wild-type, F₁ and F₂ isolates were maintained on Ws-eds1 (Parker et al. 1996). The sporulation was assessed as described (Tör et al. 2002).

Generation of the Hpa-Cala2 × Hpa-Noks1 cross and F₂ mapping population.

Spores of the two parent isolates were harvested 7 days after inoculation, and spore concentration was adjusted to 5×10^4 spores per milliliter. Equal volumes of spores from each isolate were mixed and inoculated onto 3-week-old Ws-eds1 seedlings. The pathogen was permitted to sporulate, and plants were harvested 4 weeks after inoculation to allow oospore formation. Infected plant materials were dried at room temperature and were ground to a fine powder. Oospore-containing powder was sprinkled onto soil, Ws-eds1 seeds were then planted onto the same soil and were incubated at 4°C for a week. Between 4 and 10 days, seedlings were inspected for sporulation, individual seedlings bearing sporophores were removed, and the spores were suspended in 50 µl of water. These spores were

then bulked-up on Ws-eds1 seedlings and PCR analyses were performed to identify F₁ isolates. Ppat/2/gen10/F 5'-CTTC CAATTACCCGCCATCGAGATGCCG-3' and Ppat/2/gen10/R 5'-CTGGTCAATCTGGCGGAGACATCAAAG-3' primers were used to amplify DNA from parents as well as from putative F₁ isolates, and the product was digested with *Bs*II to reveal the polymorphism.

The F₁ identified was confirmed with other PCR markers, including AFLP primers, and was designated CaNo F₁, which was then single-spored, bulked-up on Ws-eds1 plants, and allowed to form oospores. Segregating 50 random CaNo F₂ isolates were obtained from Ws-eds1 seedlings that were inoculated with oospores derived from selfed F₁ isolate.

Segregation analysis and map-based cloning of ATR5^{Emoy2}.

CaNo F₂ isolates were single-spored, were bulked-up on Ws-eds1, and were tested on Ler-0, Ler-rpp5, Col-5, and Ws-eds1 to determine the genetic nature of ATR5. Bulk segregant analysis using the AFLP method was employed to clone ATR5.

Two different bulks were constructed from the CaNo F₂ individuals (15 F₂ with ATR5/± and 15 with *atr5/atr5* genotypes), and AFLP was carried out with 128 *Eco*RI and *Mse*I primer pairs as described (Rehmany et al. 2003). Putative linked AFLP markers were tested on individuals from which the bulks were constructed. If the linkage was confirmed, these AFLP markers were converted to CAPS markers and were used for screening further additional F₂ lines to identify recombinants as well as for screening the *H. arabidopsidis* BAC library. Ler-0 seedlings were used as bait instead of Ws-eds1 to produce additional 110 CaNo F₂ individuals with *atr5/atr5* genotypes. Generation of the cross between Hpa-Maks9 and Hpa-Emoy2 and the isolation of F₂ mapping lines have been described elsewhere (Gunn et al. 2002). The *H. arabidopsidis* Hpa-Emoy2 BAC library (Rehmany et al. 2003) filters were

Fig. 9. Phylogenetic tree of the ATR5^{Emoy2} paralogs and homologs from *Hyaloperonospora arabidopsidis*. Neighbor-joining tree generated from the alignment of entire amino-acid sequences. Numbers indicate percentage bootstrapping values.

screened with the CAPS markers, using standard methods for radioactive labeling and hybridization. BAC clones identified from this screen and the available BAC contig data (Wellcome Trust Sanger Center, Cambridge) were used to establish an overlapping BAC contig spanning the *ATR5* locus. Recombinant CaNo F₂ lines from either side of the interval were used to narrow the interval down to a single BAC clone, HPAC23F10. This BAC clone was then sequenced, and putative candidate genes within the interval were identified using the Artemis sequence annotation tool (Wellcome Trust Sanger Institute).

Bombardment assays.

Candidate genes were cloned into the plant expression vector pEarleyGate100 (Earley et al. 2006) using the Gateway recombination system (Invitrogen, Carlsbad, CA, U.S.A.). Full-length *ATR5* candidate alleles were amplified from Noks1- and Emoy2-infected *A. thaliana* tissues using primers 5'-AACGTAACCGCCAAAGATGC-3' and 5'-GCTCTCGAGTTAGTTAGGAGTGACG-3' and were cloned into pCR8/GW/TOPO entry vector (Invitrogen) by TA cloning. *ATR5* alleles were transferred to the binary vector pEarleyGate100 (Earley et al. 2006) that has the constitutive 35S *Cauliflower mosaic virus* promoter by LR recombination (Invitrogen). Expression clones lacking the signal peptide and N-terminal region up to and including the EER motif were similarly produced using primers incorporating an ATG start codon (underlined) 5'-CAATATGCGTCACGTGAACCTCTCGG-3' and 5'-ATGACGCC TGGGAATCGTTTAT-3', respectively, and primer 5'-GCTCTCGAGTTAGTTAGGAGTGACG-3'. Each expression clone was sequence-verified before use in cobombardment assays.

Cobombardment assays were performed as described previously (Rehmany et al. 2005). Briefly, *A. thaliana* plants were grown with 10 h of light at 20°C until 8 weeks old. Detached leaves were placed on a Whatman 3MM paper supported on 1% agar in a petri dish. DNA (1 µg) from each pEarleyGate100 expression clone were mixed with pEarleyGate100:GUS (1 µg) and were coated onto M17 tungsten particles (Bio-Rad, Hercules, CA, U.S.A.). A control of pEarleyGate100:GUS and pEarleyGate100 (empty vector) were also prepared in the same way. Each mixture was used to make six replica bombardments. Bombardments were performed using a Bio-Rad PDS-1000 (He) apparatus with 1,100-p.s.i. rupture disks, as per manufacturer's instructions. For each replicate, a leaf from both test and control plant genotypes were cobombarded together in a single shot. Petri dishes with bombarded leaves were wrapped with parafilm and were incubated at 25°C for 20 h. Histochemical GUS staining was performed using 5-bromo-4-chloro-3-indolyl glucuronide at 37°C for 4 h. Tissues were cleared with methanol before scoring leaves for blue-stained cells, using a light microscope. Experiments were repeated six times.

Southern blotting.

Genomic DNA was isolated from a Ws-eds seedling infected with *H. arabidopsidis* 7 days postinoculation, using a cetyltrimethylammonium bromide extraction protocol based on that of Rogers and Bendich (1985). DNA was digested and separated on a 0.8% agarose gel before alkaline transfer to Hybond-N+ membrane (GE Healthcare, Piscataway, NJ, U.S.A.). Membranes were probed with a ³²P-labeled probe derived from *ATR5*^{Emoy2}.

Expression analysis.

Total RNA was isolated from uninfected Ws-eds1 plants or plants infected with Hpa-Emoy2 at 1, 2, 4, and 7 days after inoculation. RT-PCR analyses were performed with *ATR5* gene-specific primers as previously specified, using 0.5 µg of

total RNA as template. The PCR products were separated on a 1.5% agarose gel and were stained with ethidium bromide. The products were sequence verified. *Hpa-actin* gene was used as a control in RT-PCR assays.

RACE and DNA sequencing.

Total RNA was isolated from infected and noninfected cotyledons, using TRIzol reagent (Invitrogen), and was concentrated using the RNeasy plant mini kit (Qiagen Ltd., West Sussex, U.K.) according to the manufacturer's instructions. RACE was performed with the GeneRacer kit, per manufacturer's instructions (Invitrogen, Glasgow, U.K.) with modification. First-strand cDNA synthesis was performed using the GeneRacer oligo dT provided with the Superscript III RT module (5'-GCTGTCAACGATACGCTACGTAACGGCATGACAGT G(T)24-3'). Nested 3' PCR was carried out with two gene specific primers (GSP), GSP1 (5' CTCGTGGTTTCCACCGCGA TTCAAG-3') and GSP2 (5'-CCGGAGGAATGCGAAACCCG GTATTA-3') against the GeneRacer 3' and the GeneRacer 3' nested primers, respectively. For 5' RACE PCR, nested PCR was performed with two GSP, GSP1 (5'-CCCGTTTCCTTCA AACGCACATCCA-3'), and then, GSP2 (5'-ACGGTGTGTG TATTGAAGGCGCAAGA-3') against the GeneRacer 5' and GeneRacer 5' nested primers, respectively. A touchdown PCR regime was employed at 95°C for 30 s, 72°C for 3 min for 5 cycles, 95°C for 30 s, 70°C for 30 s, 72°C for 3 min for 5 cycles and 95°C for 30 s, 68°C for 30 s, 72°C for 3 min for 25 cycles, followed by 10 min extension at 72°C, using KOD polymerase (Novagen, Merck Chemicals, Nottingham, U.K.). The PCR products were analyzed by gel electrophoresis and were cloned into pCR4-TOPO vector (Invitrogen, Glasgow, U.K.). Both DNA strands were sequenced, in all cases using the BigDye termination kit (Applied Biosystems, Foster City, CA, U.S.A.), and were separated on an ABI 377 sequencer. Cloned products were sequenced using the universal M13 primers. The BAC clone was sequenced by the Genome Sequencing Center, Washington University (St. Louis).

Agrobacterium-mediated plant transformation.

Full-length candidate genes, *ATR5*^{Emoy2}, *ATR5L1*^{Emoy2}, and *ATR5L2*^{Emoy2} and *ATR5L*^{Noks1} were cloned into Gateway compatible plant transformation vector pBAV150 (Vinatzer et al. 2006), using Gateway cloning technology (Invitrogen, Glasgow, U.K.). Ligated products were electroporated into *Escherichia coli* DH10B, and positive clones were identified by PCR and verified by sequencing. The constructs were introduced into *Agrobacterium tumefaciens* GV3101 by electroporation, and Ler-0 plants were then transformed with each of the constructs. Transformants were selected by spraying 7-day-old plants grown in soil with 0.04% BASTA (Agrevo, Cheshire, U.K.) and were allowed to self-pollinate. T₂ seeds were grown in soil with Basta and were sprayed 7 days after germination with 30 µM dexamethasone to induce gene expression.

Bioinformatics.

The Virginia Bioinformatic Institute Microbial and Broad Institute *Phytophthora infestans* databases were used for contig information on *H. arabidopsidis* as well as for searching for other oomycete genes. Several web servers, including InterPro, SMART, PFAM, and I-TASSER were employed for the analysis of the *ATR5* protein. Several custom Perl scripts were used in the analysis of *ATR5*-like genes. The *H. arabidopsidis* genome (version 2.0) was used to search for all possible open reading frames (ORF), considering all methionines as potential start codons. Local copies of SignalP V3.0 and TMHMM were obtained, and each predicted ORF was tested for the presence of a signal peptide and transmembrane helices. A Perl script

using regular expressions and pattern matching was then used to select ORF from this dataset that contained the motif EER but not R[X]LR within the first 100 amino acids of the mature peptide. Primer designs, in silico digests, and comparison of genomic and full-length cDNA sequences of *ATR5* were performed using CLC Combined Workbench (CLC Bio, Aarhus, Denmark).

***Pseudomonas syringae* pv. *tomato* DC3000 pathology experiments.**

Mobilization of *ATR5-ΔSP* family effectors into pEDV vectors, plant inoculations with DC3000, and in planta bacterial growth assays were performed as described (Sohn et al. 2007).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Broad Institute *Phytophthora infestans* database:
www.broad.mit.edu/annotation/genome/phytophthora_infestans/MultiHome.html

European Arabidopsis Stock Centre website: arabidopsis.info

European Bioinformatics Institute InterPro website: www.ebi.ac.uk

PFAM database: pfam.sanger.ac.uk

The SignalP V3.0 website: www.cbs.dtu.dk/services/SignalP

The SMART database: smart.embl-heidelberg.de

The TMHMM prediction server: www.cbs.dtu.dk/services/TMHMM

Virginia Bioinformatic Institute microbial database: vmd.vbi.vt.edu

The Washington University Genome Sequencing Center website:
genome.wustl.edu