Arabidopsis Downy Mildew Resistance Gene *RPP27* Encodes a Receptor-Like Protein Similar to *CLAVATA2* and Tomato *Cf-9*[1]

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The Arabidopsis L*er-RPP27* gene confers *AtSgt1b*-independent resistance to downy mildew (*Peronospora parasitica*) isolate Hiks1. The *RPP27* locus was mapped to a four-bacterial artificial chromosome interval on chromosome 1 from genetic analysis of a cross between the enhanced susceptibility mutant Col-*edm1* (Col-*sgt1*) and Landsberg *erecta* (L*er*-0). A *Cf*-like candidate gene in this interval was PCR amplified from L*er*-0 and transformed into mutant Col-*rpp7.1* plants. Homozygous transgenic lines conferred resistance to Hiks1 and at least four L*er*-0 avirulent/Columbia-0 (Col-0) virulent isolates of downy mildew pathogen. A full-length *RPP27* cDNA was isolated, and analysis of the deduced amino acid sequences showed that the gene encodes a receptor-like protein (RLP) with a distinct domain structure, composed of a signal peptide followed by extracellular Leu-rich repeats, a membrane spanning region, and a short cytoplasmic carboxyl domain. *RPP27* is the first RLP-encoding gene to be implicated in disease resistance in Arabidopsis, enabling the deployment of Arabidopsis techniques to investigate the mechanisms of RLP function. Homology searches of the Arabidopsis genome, using the *RPP27*, *Cf-9*, and *Cf-2* protein sequences as a starting point, identify 59 RLPs, including the already known *CLAVATA2* and *TOO MANY MOUTHS* genes. A combination of sequence and phylogenetic analysis of these predicted RLPs reveals conserved structural features of the family.

A wide range of parasites, including viruses, bacteria, fungi, nematodes, and insects, exploit plants as a source of food and shelter. Plants have evolved mechanisms to recognize the potential colonists and defend themselves. The defense is often activated by the direct or indirect interaction of the disease resistance (*R*) gene in the plant and the avirulence (*Avr*) gene in the pathogen (Dangl and Jones, 2001; Holub, 2001). The absence of either of these genes results in infection by the pathogen.

To date, numerous *R* genes have been cloned from a wide range of plant species, including Arabidopsis, flax (*Linum usitatissimum*), tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), sugar beet (*Beta vulgaris*), apple (*Malus domestica*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), and maize (*Zea mays*). Their structural and functional comparisons have been well documented, revealing several different classes (Hammond-Kosack and Jones, 1997; Hammond-Kosack and Parker, 2003; Tör et al., 2003). The largest group of *R* genes encodes cytoplasmically localized proteins that contain a central nucleotide binding (NB) site and a carboxyl Leu-rich repeat (LRR) domain (NB-LRR genes). This group can be further subdivided into two major subclasses: those having an amino-terminal coiled-coil (CC) domain (CC-NB-LRR) and those containing an amino-terminal domain resembling the cytoplasmic signaling domain of the Toll and Interleukin-1 (TIR) transmembrane receptors (TIR-NB-LRR). The CC-NB-LRR subclass includes examples such as the Arabidopsis *RPS2* (Mindrinos et al., 1994) and *RPM1* (Grant et al., 1995) genes conferring bacterial resistance, *RPP13* (Bittner-Eddy et al., 2000) and *RPP8* (McDowell et al., 1998) conferring downy mildew (*Peronospora parasitica*) resistance, and *HRT* (Cooley et al., 2000) conferring viral resistance from the same locus as *RPP8*. The TIR-NB-LRR subclass includes genes such as the tobacco *N* (Whitham et al., 1994) gene for viral resistance, the flax *L6* (Lawrence et al., 1995) gene for rust resistance, and the Arabidopsis *RPP5* (Parker et al., 1997) and *RPP1* (Botella et al., 1998) genes for downy mildew resistance. Sequencing of the complete genome of Arabidopsis has revealed approximately 149 NB-LRR genes (Meyers et al., 2003).

The second group contains the cytoplasmic Ser/Thr kinase and has been represented by *PTO* (Martin et al., 1993), which confers resistance to the bacterial pathogen *Pseudomonas syringae* pv *tomato*.

The third group of *R* genes encodes the receptor-like kinases (RLKs). The characteristic features of these

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proteins are an extracellular LRR domain with a single transmembrane spanning region and a cytoplasmic kinase domain. This group contains the rice *Xa21* gene (Song et al., 1995), which confers resistance to bacterial pathogen *Xanthomonas oryzae* pv o*ryzae*.

Receptor-like proteins (RLPs) comprise the fourth group of *R* genes. These are similar to RLK genes in that they encode extracellular LRRs and a C-terminal membrane anchor but lack the cytoplasmic kinase domain. Members of this group include the tomato *Cf-2*, *Cf-4*, *Cf-5*, and *Cf-9* genes conferring resistance to the fungal pathogen *Cladosporium fulvum* (Jones et al., 1994; Dixon et al., 1996), the tomato *Ve* genes for Verticillium resistance (Kawchuk et al., 2001), and the apple *HcrVf2* gene for resistance to *Venturia inequalis* (Belfanti et al., 2004).

To date, all cloned Arabidopsis *R* genes conferring resistance to the oomycete downy mildew pathogen belong to the NB-LRR class. Here, we describe the molecular cloning of the Arabidopsis *RPP27* gene that confers resistance to several isolates of *P. parasitica* and encodes an RLP. The coding sequence of this gene predicts a protein with topological features similar to Arabidopsis *CLAVATA2* (*CLV2*) and tomato *Cf-9*.

RESULTS

Identification and Isolation of the *RPP27* Gene

RPP27 was previously identified as a gene in the accession Landsberg *erecta* (Ler-0) that functions independently of *AtSGT1b* and confers resistance to Peronospora isolate Hiks1. Generation of mapping material and segregation data from the cross between a 35-kb deletion mutant Col-*sgt1b* and L*er*-0 were described previously (Tör et al., 2002). Linkage of *RPP27* to the molecular marker g4026 on chromosome 1 was determined in this previous work. Using 410 Hiks1 susceptible F_2 families, *RPP27* was mapped further between two PCR markers, Nga280 and F12M16 (Fig. 1A). Two new markers, AC1 and AC2, were generated from the sequence information of two bacterial artificial chromosomes (BACs), T18A20 and T22H22, and used to identify 14 key recombinant individuals. Four overlapping BAC clones, T18A20, F15I1, F20D21, and T22H22, span the *RPP27* interval (Fig. 1B). The sequence information and annotations of these BAC clones were examined in detail and a *Cf*-like gene on the BAC clone F20D21 (F20D21. 29) was identified. Another PCR marker, CFL, was generated

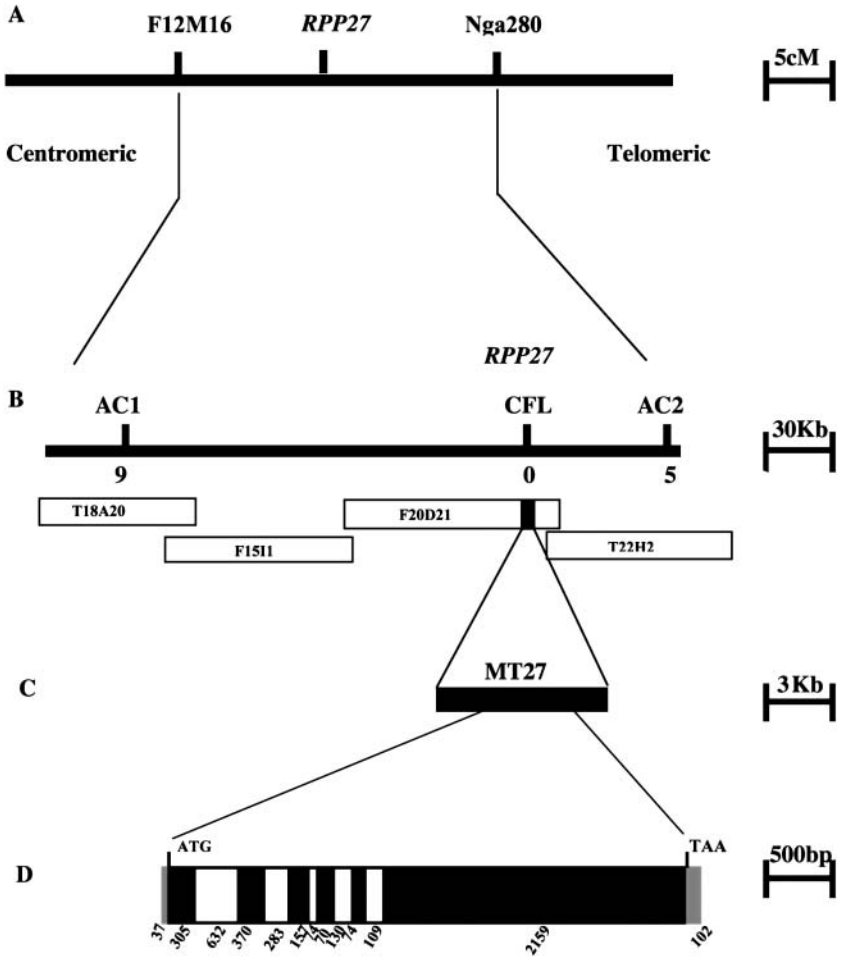

Figure 1. Map-based cloning of *RPP27*. Genetic map of *RPP27* locus showing the molecular markers F12M16 and Nga280 that were initially used to define the mapping interval. cM, Centimorgan (A). B, The BAC contig spanning the *RPP27* locus that was fine mapped with the markers shown above the bar. The numbers of recombinant individuals identified with the markers are shown below the bar. The black bar on the BAC clone F20D21 represents the *Cf*-like gene. C, This region was amplified from L*er*-0, inserted into a binary vector to produce MT27, which was then introduced into Col-*rpp7.1* plants. The cDNA was obtained and compared with genomic DNA to reveal the structure of the *RPP27* gene. Untranslated regions are shown as gray, exons are shown as black, and introns are shown as white bars. D, Numbers below indicate the size of untranslated regions, exons, and introns.

from the sequence of this gene and used for mapping. This marker cosegregated with the *RPP27* phenotypic data (Fig. 1B), indicating that this gene was a strong candidate for *RPP27.* A PCR-cloning approach was then taken to clone the corresponding region of this *Cf*-like gene from L*er*-0. Using the available sequence information of BAC clones and accommodating possible misannotation of the gene, a fragment of 6,393 bp (encompassing a 2,034-bp promoter region, 3,366-bp coding region, and 993 bp beyond the stop codon) was targeted to be cloned from L*er*-0. This region was PCR amplified using a proofreading DNA polymerase and cloned into a binary vector to produce the construct MT27 (Fig. 1C). The cloned insert and the corresponding genomic region from the L*er*-0 *RPP27* locus were verified by DNA sequencing.

Transgenic Complementation of *RPP27* Function

The wild-type Columbia-0 (Col-0) carries the *RPP7* gene that recognizes the downy mildew isolate Hiks1 (Holub et al., 1994; McDowell et al., 2000). Therefore, the construct MT27 was transformed into Col-*rpp7.1* mutant plants to confirm that the putative *Cf*-like gene corresponds to the *RPP27* gene. Fifteen independent transgenic T_2 seedlings were assessed for resistance against Hiks1. All the lines segregated for the *RPP27* and *rpp27* phenotype (mostly 3:1), correlating with basta resistance and sensitivity, respectively, indicating that the cloned DNA fragment carries the *RPP27* gene.

Homozygous T_3 families were then obtained from these transgenic lines and examined for their capacity to generate H_2O_2 24 h after inoculation with Hiks1 using 3,3′-diaminobenzidine (DAB). This compound captures H_2O_2 and forms a reddish brown polymer at sites of peroxidase activity (Thordal-Christensen et al., 1997), thus providing a means for detecting an oxidative burst in host cells surrounding penetrating hyphae. More than 100 cotyledons from nontransgenic Col-*rpp7.1* and from lines transformed with *RPP27* were examined. No DAB staining was observed around the infection sites of cotyledons from nontransformed plants. Instead, normal pathogen growth was observed (Fig. 2A). In the cotyledons of *RPP27*-transformed plants, DAB staining was observed around the infection site mainly in one or two cells per infection site (Fig. 2B). However, the DAB staining observed in the transgenic cotyledons was not as strong as we reported previously for *RPP7*-mediated resistance (Tör et al., 2002), suggesting that L*er-RPP27* confers partial resistance to Hiks1.

We examined the pathogen development and interaction phenotype in detail. Three days after inoculation, normal pathogen development was observed in the cotyledons of control nontransformed seedlings,

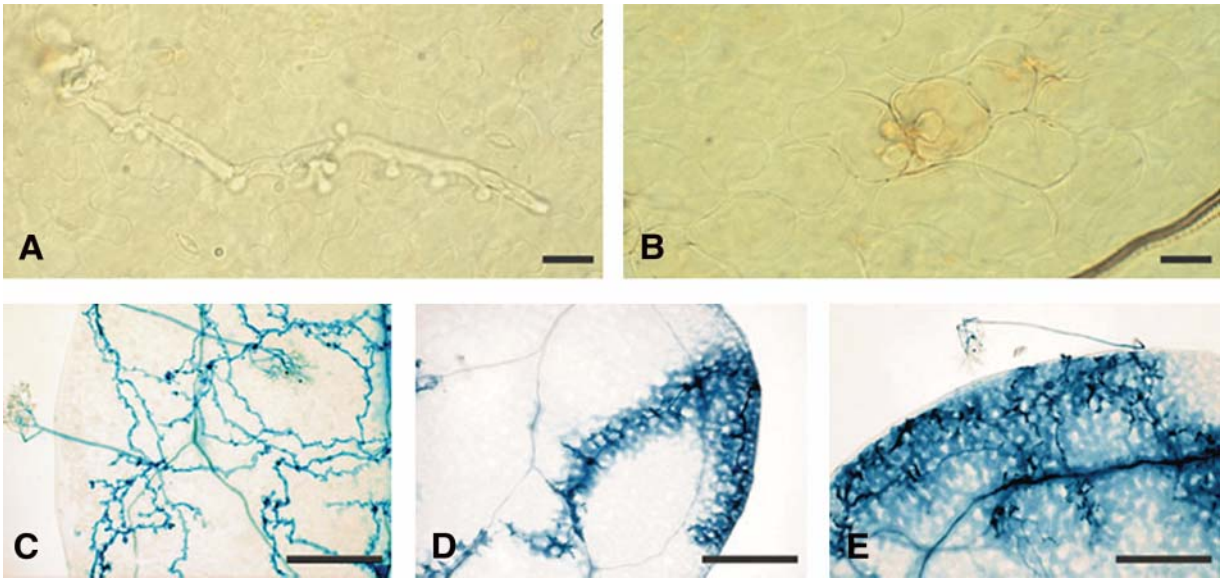

Figure 2. Pathogen development and interaction phenotypes of transformed and nontransformed plants inoculated with downy mildew isolate Hiks1. Cotyledons stained with DAB 1 d after inoculation and examined under a light microscope for H_2O_2 accumulation are shown in A and B (bar = 50 μm). A, Normal pathogen development and no H_2O_2 detection was observed in Col-*rpp1.1*. B, Accumulation of H_2O_2 was detected with DAB staining around the Hiks1 penetration sites in Col-*rpp7* transformed with *RPP27*. Cotyledons stained with lactophenol-trypan blue 3 d and 7 d after inoculation and viewed under a light microscope to reveal pathogen mycelium and necrotic plant cells are shown in C and E (bar = 10 μm). C, Col-*rpp7.1*, shown with normal pathogen development, fully susceptible to Hiks1 3 d after inoculation. D, Col-*rpp7.1*::L*er-RPP27*, showing mycelium growth beyond the penetration site but surrounded by a trail of necrotic plant cells 3 d after inoculation. E, Col-*rpp7.1*::L*er-RPP27*, showing extensive mycelial growth and trailing necrosis with a conidiophore development, which was observed occasionally 7 d after inoculation.

and host cells appeared to be intact (Fig. 2C). However, less pathogen growth and trailing necrosis was observed in colonized areas of transgenic seedlings (Fig. 2D). Trailing necrosis was more extensive in transgenic seedlings 7 d after inoculation, and occasionally the pathogen produced conidiophores (Fig. 2E).

RPP27 Recognizes More Than One Isolate of Downy Mildew

We extended the analysis to see whether *RPP27* confers resistance to any isolate of downy mildew other than Hiks1. Col-0 compatible isolates but L*er*-0 incompatible isolates, including Aswa1, Edco1, Emco2, Emco5, Emwa2, Goco1, Gowa1, Maks9, Noco2, and Noks1, were used to inoculate homozygous transgenic lines along with controls, including wild-type resistant L*er*-0, wild-type susceptible Col-0, and nontransgenic mutant Col-*rpp7.1*. Asexual sporulation was measured by quantifying sporangiophore production as described previously (Tör et al., 2002) and was compared with the controls. Control seedlings Col-0 and Col-*rpp7.1* were susceptible to all the isolates tested. However, *RPP27* transgenic Col-*rpp7.1* was susceptible to Aswa1, Edco1, Emwa2, Gowa1, and Maks9 but resistant to Emco2, Goco1, Noco2, and Noks1. Interestingly, *RPP27* transgenic Col-*rpp7.1* inoculated with Emco5 showed the phenotype of low sporulation (L3). The results are summarized in Table I.

Sequence Analysis of the *RPP27* Gene and Transcript

The construct MT27 that carries the L*er*-0 genomic DNA fragment was sequenced using a primer walking strategy and shown to contain a 6,461-bp insert. This region has been annotated in the EMBL database in the original BAC clone as gene F20D21.29, encoding a protein of 818 amino acids similar to *Cf*-like genes in tomato. However, this region has been annotated in the Munich Information Center for Protein Sequences (MIPS) database as two genes, At1g54470 encoding an 112-amino acid hypothetical protein and At1g54480 encoding a 550-amino acid protein similar to disease resistance genes. Similarly, The Institute for Genomic Research (TIGR; Rockville) database showed two genes, At1g54470 encoding a 113-amino acid hypothetical protein and an At1g54480 encoding a 551-amino acid protein similar to the LRR protein family. Extensive database searches revealed no expressed sequence tags corresponding to the *RPP27* genomic sequence.

Since there were no ESTs for *RPP27* and the MIPS and TIGR annotations of the region disagreed, we carried out several reverse transcription (RT)-PCR experiments with RNA isolated from Hiks1 infected and noninfected seedlings from Col-0 and L*er*-0 to determine the expression level of *RPP27*. A very low level of expression of *RPP27* was observed in tissues of both Col-0 and L*er*-0 (data not shown). The 3′ RACE from L*er*-0 revealed a predicted stop codon, a 3′ untranslated region of 102 bp, and a polyadenylation site. The 5′ end of the transcript from L*er*-0 indicated a single major transcription site 37 bp upstream of the predicted ATG. Based on the cDNA 5′ RACE and 3′ poly(A) site, the *RPP27* transcript is found to be 3,274 bases long. Comparison of the genomic and the cDNA sequences allowed us to define six exons and five introns (Fig. 1D).

When the RPP27 genomic sequence in the construct MT27 was compared with the corresponding region of Col-0 on the BAC clone F20D21, we observed 11 sites to be polymorphic. Of these 11 sites, six were single nucleotide polymorphisms and the other five were insertions/deletions (INDELs) (Table II). Six of these polymorphic sites were in the promoter region; three were in introns; one (a large deletion of 68 nucleotides

Table I. *Phenotypes of four Arabidopsis accessions used to determine whether the RPP27 allele from Ler confers resistance to downy mildew isolate Hiks1 and to 10 other isolates that are virulent in Col and avirulent in Ler-0*

Downy Mildew Isolate	Arabidopsis Accession			
	Col-0	Col-*rpp7.1*	L*er*-0	Col-*rpp7.1*::*Ler-RPP27*
Hiks1	N (*RPP7*)	H	N (*RPP7* and *RPP27*)	N
Aswa1	H	H	L5 (n.d.)	H
Edco1	H	H	N (n.d.)	H
Emwa2	H	H	N (n.d.)	H
Gowa1	H	H	N (n.d.)	H
Maks9	H	H	L2 (n.d.)	H
Emco2	H	H	N (n.d.)	N
Emco5	H	H	N (*RPP8*)	L3
Goco1	H	H	N (n.d.)	N
Noco2	H	H	N (*RPP5*)	N
Noks1	H	H	N (*RPP5*)	N

Approximately 50 7-d-old seedlings were spray inoculated with downy mildew conidiospores. Asexual sporulation was quantified by counting sporangiophores 7 d after inoculation as described previously (Tör et al., 2002) and summarized as follows: N, no sporulation; L, low sporulation (1–10 sporangiophores per cotyledon; the mean is indicated by a number); medium sporulation (11–20 sporangiophores); and H, heavy sporulation (mean > 20 sporangiophores). *R* genes that have been molecularly characterized in the wild type accessions are indicated in parentheses (see Holub, 2001). n.d., not determined.

Table II. *Sequence polymorphisms in the RPP27 region between Col-0 and Ler-0*

Nucleotide Position[a]	Col-0	Ler-0
−1,044	A	G
−1,024	A	−
−949	A	G
−877	A	−
−512	−	G
−412	T	C
412[b]	C	T
486[b]	A	G
1,799[b]	T	C
2,574[c]	−	CGAGTACCAGAAAAACTTGCGTCTAGT-TGATCTATCCAACAACAGATTATCTG-GAAACCTACCTACAT
4,576	−	T

[a]Nucleotide positions are numbered with respect to translational start site. [b]Polymorphism is in the intron. [c]Polymorphism is in the exon. −, Deletion.

in Col-0) was in the largest exon; and one came just after the 3′ untranslated region. The large INDEL caused a frameshift in the largest exon resulting in a premature stop codon. These polymorphisms therefore have a significant effect on the predicted amino acid sequences of RPP27 protein from Col-0 accession and may also have played a role in the misannotation of the region described above.

Predicted *RPP27* Protein Structure

The open reading frame of the *RPP27* gene encodes a predicted protein of 1,044 amino acids (molecular mass of 116.9 kD) with structural similarity to other RLPs, including Arabidopsis *CLV2* (Jeong et al., 1999) and *TOO MANY MOUTHS* (*TMM*; Nadeau and Sack, 2002), and tomato *Cf-9* (Jones et al., 1994) and *Cf-2* (Dixon et al., 1996). A variety of bioinformatics approaches were used to predict the structure of *RPP27* (see "Materials and Methods"). As with the tomato *Cf-9* and Arabidopsis *CLV2* protein, *RPP27* can be divided into seven domains (Fig. 3): an initial signal peptide (domain A, M1-S20), followed by an LRR (domain B, Q21-S143), an LRR (domain C, I144-G946), a variable region (domain D, residues R947-C972), an acidic region (domain E, D973-L997), a predicted transmembrane domain (domain F, A998-F1020), and a short cytoplasmic tail (domain G, D1021-P1044).

Domain C constitutes the majority of the predicted *RPP27* protein and consists of 30 imperfect copies of extracellular LRRs with a consensus sequence of LxxLxxLxxLxLxxNxLSGxIPxx. This region has an island of variable and mostly hydrophobic sequences between positions 789F and 855D that matches similar regions in other RLPs; the exact structure of this region cannot be specified using sequence analysis methods. It is possible that this region provides a flexible hinge to the two flanking LRR domains (C1 and C3), allowing them to articulate relative to each other.

A MKTNFVILLLLLCVFAISPS

B1 QQEEINQHNPGIYHQKLLYKVQQ
WRTSLKESNSVELKLSLAAIVA

B2 GVLYFLAALISSACGIGSGGLFI

B3 PITTLVSRLDLKTGKRFLGQYLIWVILLLGQ
LHECKSCIEKERVALLDFKKYWMS

C1 ITQESDLDYVFPTWNNDTKSDCCQWESIMC
NPTSGRLIRLHVGASNLKENSLLNISL
LHPFEEVRSLELSAG-LNGFVDNVEGYKS
LRKLKNLEILDLSYNNRFNNNILPF
INAATSLTSLSLQNNSMEGPFPFEE
IKDLTNLKLLDLSRNILKGPMQG
LTHLKKLKALDLSNNVFSSIMELQV
VCEMKNLWELDLRENKFVGQLPLC
LGRLNKLRVLDLSSNQLNGNLPST
FNRLESLEYLSLLDNNFTGFFSFDP
LANLTKLKVFKLSSTSDMLQIKTESE
PKYQFQLSVVVIRVCSLEK-IPSF
L*EYQKNLRLVDLSNNRLSGNLPTWL
LANNPELKVLQLQDNLFTIFQMP
ATIVHELQFLDFSVNDISGLLPDNI
GYALPNLLRMNGSRNGFQGHLPSS
MGEMVNITSLDLSYNNFSGKLPRRF
VTGCFSLKHLKLSHNNFSGHFLPR
ETSFTSLEELRVDSNSFTGKIGVGL
LSSNTTLSVLDMSNNFLTGDIPSW
MSNLSGLTILSISNNFLEGTIPPS
LLAIGFLSLIDLSGNLLSGSLPS
RVGGEFGIKLFLHDNMLTGPIP
DTLLEKVQILDLRYNQLSGSIPQ
FVNTESIYILLMKGNNLTGSMSRQ
LCDLRNIRLLDLSDNKLNGFIPSC

C2 LYNLSFGPEDTNSYVGTAITKITPFKFYESTFV
VEDFVVISSSFQEIEIKFSMKRRYDSYF
GATEFN

C3 NDVLDYMYGMDLSSNELSGVIPAE
LGSLSKLRVMNLSCNFLSSSIPSS
FSNLKDIESLDLSHNMLQGSIPQQ
LTNLSSLVVFDVSYNNLSGIIPQG

D RQFNTFDEKSYLGNPLLCGPPTNRSC

E DAKKTSDESENGGEEEDDEAPVDML

F AFYFSSASTYVTTLIGIFILMCF

G DCPLRRAWLRIVDASIASVKSMLP

LxxLxxLxxLxLxxNxLSGxIPxx

Figure 3. Predicted domain structure of *RPP27*. Domains A to G correspond with previous diagrams of *Cf-9* (Jones et al., 1994; see text for descriptions). The B region is divided into B1, B2, and B3 to show the presence of its predicted TM region (B2, underlined). The site of the large deletion relative to L*er*-0 is marked with an asterisk. The LRR consensus sequence appears boxed and aligned below the full sequence. The C region is shown divided into C1 (main LRR block), C2 (non-LRR island), and C3. The presence of the island of non-LRR sequence before the final four LRRs is a common element in RLPs and may be a structural hinge that allows the C1 block to adopt its correct conformation. The C3 region is highly conserved within the family and may be required for multiprotein complex formation.

Despite similarities between *RPP27* and the product of *Cf* genes (particularly *Cf-2* and *Cf-9*), there are two striking differences. First, relative to the tomato *Cf* proteins and the vast majority of other Arabidopsis RLPs, *RPP27* has a highly divergent amino-terminal B domain, with an initial region of approximately 120 amino acids preceding a region of significant homology with *Cf-2* and *Cf-9*.

Second, in contrast to all other plant RLPs identified thus far, which are predicted to have a single transmembrane domain at the C terminus, transmembrane prediction using the TMHMM server identifies two putative transmembrane (TM) domains in *RPP27*, one at the C terminus and one in the variable N-terminal domain (66G–88I). However, since transmembrane prediction algorithms are known to be misled by hydrophobic stretches (Chen et al., 2002), we examined the N-terminal region in depth. In contrast to the C-terminal transmembrane domains, which share a recognizable sequence signature and conserved residues, the N-terminal predicted transmembrane domain of *RPP27* does not match any characterized transmembrane domains in any protein. In addition, analyses of other LRR proteins show both a high degree of sequence divergence across LRRs, and some regions of LRR proteins contain hypervariable stretches. These points, in combination with the lack of identified proteins with what would represent an entirely novel fold, suggest to us that this region of *RPP27* is simply somewhat variable and hydrophobic and that it is not transmembrane. However, the structure of this region is extremely challenging to homology-based methods of structure prediction and, since we cannot rule out the possibility that this region contains a transmembrane domain, we plan to explore this experimentally.

RLP Family Analysis

The consensus RLP fold consists of multiple LRRs, followed by a transmembrane domain and a short cytoplasmic tail. Because LRRs are often found in proteins with non-RLP folds, sequence-based methods of homolog detection can inadvertently include many non-RLPs in database searches. Similarly, transmembrane prediction tools can overpredict TM domains in hydrophobic stretches. To discriminate true RLPs from sequences with different overall folds, we employed a multistep analysis. First, we used *RPP27*, *Cf-2*, and *Cf-9* as BLAST queries against Arabidopsis. We then scored Arabidopsis proteins with a hidden Markov model (HMM; Krogh et al., 1994) that we constructed to specifically match the RLP topology. Sequences with significant BLAST e-values or strong scores against our HMM were examined and found to include many RLKs and other non-RLP proteins. We scored these proteins against PFAM HMMs and removed any proteins with e-values less than 0.5 to any non-LRR PFAM HMMs. Remaining proteins were submitted to the TMHMM transmembrane prediction server (Krogh et al., 2001). Proteins with no predicted transmembrane domains at the C terminus (or no very close homologs with a predicted transmembrane domain) were removed from the set, and the remainder was multiple aligned. Examination of the multiple sequence alignment revealed a conserved and almost entirely ungapped region of approximately 100 amino acids in domain C. Sequences not matching this region or the consensus sequence signature at the predicted transmembrane domain were removed. This procedure was intentionally stringent and identified a conservative set of 58 sequences in addition to *RPP27*. A more detailed analysis of the RLP family in plants is in progress.

We then constructed a series of multiple sequence alignments for these 59 sequences, as well as for the tomato *Cf-2* and *Cf-9* proteins. These alignments were examined and the highest quality alignment selected. From this alignment, we constructed two separate alignments, one that was essentially global (removing columns with mostly gaps) and one restricted to the C-terminal conserved domains C3 to F (Fig. 4). Phylogenetic trees were estimated using parsimony, neighbor-joining, and maximum likelihood methods, with bootstrap analysis to identify subtrees with high bootstrap support. Tree topologies were examined and found to be fairly consistent both across methods and across the two alignments; most differences were restricted to the coarse branching order in the trees. A consensus tree topology was inferred using bootstrap analysis and the PHYLIP consense software (Fig. 5).

The Arabidopsis RLP family contains 59 genes, including *RPP27*, *CLV2*, *TMM*, and 56 previously unidentified family members. These are distributed throughout the genome as follows: 17 genes, including *CLV2*, *TMM*, and *RPP27*, on chromosome 1; 12 on chromosome 2; 16 on chromosome 3; 8 on chromosome 4; and 6 on chromosome 5. The distribution is similar to NB-LRR genes, with several complex loci containing 3 or more RLPs and others that are singlets or doublets (Fig. 6). Further details are available at http://phylogenomics.berkeley.edu/PlantResistanceGene/ArabRLPs.html.

DISCUSSION

The predicted polypeptide encoded by the *RPP27* gene has structural features that indicate a receptor-like function. The signal peptide targeting the membrane at the amino terminus, the putative extracytoplasmic protein-protein interaction domain (LRR), the single transmembrane domain, and the short cytoplasmic tail present an overall structure of the large class of RLPs found in many species across the kingdom. To date, only two functional RLPs have been identified in Arabidopsis: *CLV2* and *TMM*. *CLV2* (Jeong et al., 1999) is involved in meristem and organ development and is required for the accumulation and stability of the receptor kinase *CLV1* (Clark et al., 1997).

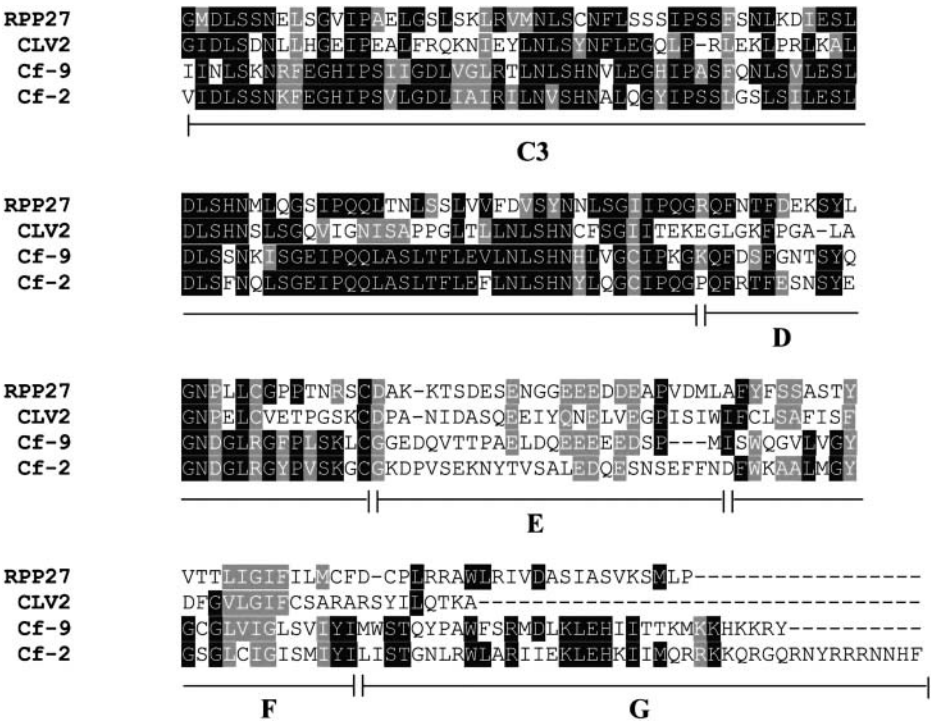

Figure 4. Multiple sequence alignment of C-terminal regions of *RPP27*, *CLV2*, *Cf-9*, and *Cf-2*. Sequences were cropped to show the C-terminal conserved region found across all Arabidopsis RLPs, which includes a section of the LRR region of the protein (domain C3) and continues past the variable (domain D), acidic (domain E), and transmembrane (domain F) domains to the C-terminal cytoplasmic peptide (domain G). Sequences were aligned using MAFFT. Identical and similar residues are displayed in black and gray boxes, respectively.

TMM (Nadeau and Sack, 2002) is involved in stomatal and epidermal development. Functional RLPs that are involved in disease resistance have been described in other plant species, including the tomato *Cf* genes (Hammond-Kosack and Jones, 1997; Joosten and De Wit, 1999) for leaf mold resistance and the *Ve* genes for Verticullium resistance (Kawchuk et al., 2001). *RPP27* provides the first example of an RLP in Arabidopsis that is associated with disease resistance. As discussed below, this discovery is particularly interesting because the pathogen downy mildew is an obligate biotrophic parasite that produces haustorial feeding structures in host cells. We have identified 59 RLPs in Arabidopsis using *RPP27*, *Cf-9*, and *Cf-2*. In a recent study, Shiu and Bleecker (2003) identified a superfamily of putative RLPs from the Arabidopsis genome. Their aim was to identify essentially all Arabidopsis proteins in Col-0 that share any recognizable sequence similarity with the extracellular portion of the RLKs. They used the extracellular domains of 35 representative RLKs to conduct BLAST searches of the Arabidopsis genome, with a cutoff value of 10 e−10. Shiu and Bleecker (2003) selected a final set of 173 proteins for cluster analysis, using the unweighted pair group method with arithmetic mean algorithm based on pairwise BLAST e-values, and produced a hierarchical tree shown in their paper. Examination of these clusters reveals a significant fraction of proteins that do not match the canonical RLP topology (transmembrane region and extracellular LRR). These proteins may well be related in some way to RLPs but are likely to have different functions. By contrast, our approach was deliberately conservative and designed to produce a set of proteins that matched the canonical RLP structure; we excluded any proteins containing structural domains not found in the RLPs and required a single transmembrane domain at the C terminus. Examination of the two sets of proteins shows that all but one of the proteins we identify as RLPs are also found by Shiu and Bleecker (2003). The exception, At1g58190, is clearly a member of the RLP class, which contains 49 LRRs, a transmembrane domain (identified by TMHMM), and a short cytoplasmic tail. Of the 117 proteins included by Shiu and Bleecker (2003) but not by us, all but four contain additional domains not found in RLPs (e.g. GDPD, B-Lectin, etc.) or are missing identifiable transmembrane segments at the C terminus. However, none of these four contain recognizable LRRs and therefore do not match the canonical RLP structure.

Phylogenetic tree estimation of these RLPs (along with the tomato *Cf* genes) place *RPP27*, *CLV2*, *TMM*, *Cf-2*, and *Cf-9* on the same branch of the evolutionary tree, suggesting that proteins involved in both disease resistance and development may have evolved from a common origin (Fig. 5). We expect that *RPP27* is not the sole example of disease resistance among these RLPs in Arabidopsis. Putative T-DNA mutants in Col-0 background exist for at least 31 of these RLPs. Challenging these mutants with Col-avirulent isolates of bacterial and filamentous pathogens such as *P. syringae* and downy mildew, respectively, may yield more information on the involvement of these RLPs in disease resistance.

The RPP27 protein has an overall topology consistent with the canonical RLP fold (Fig. 3): an amino-terminal

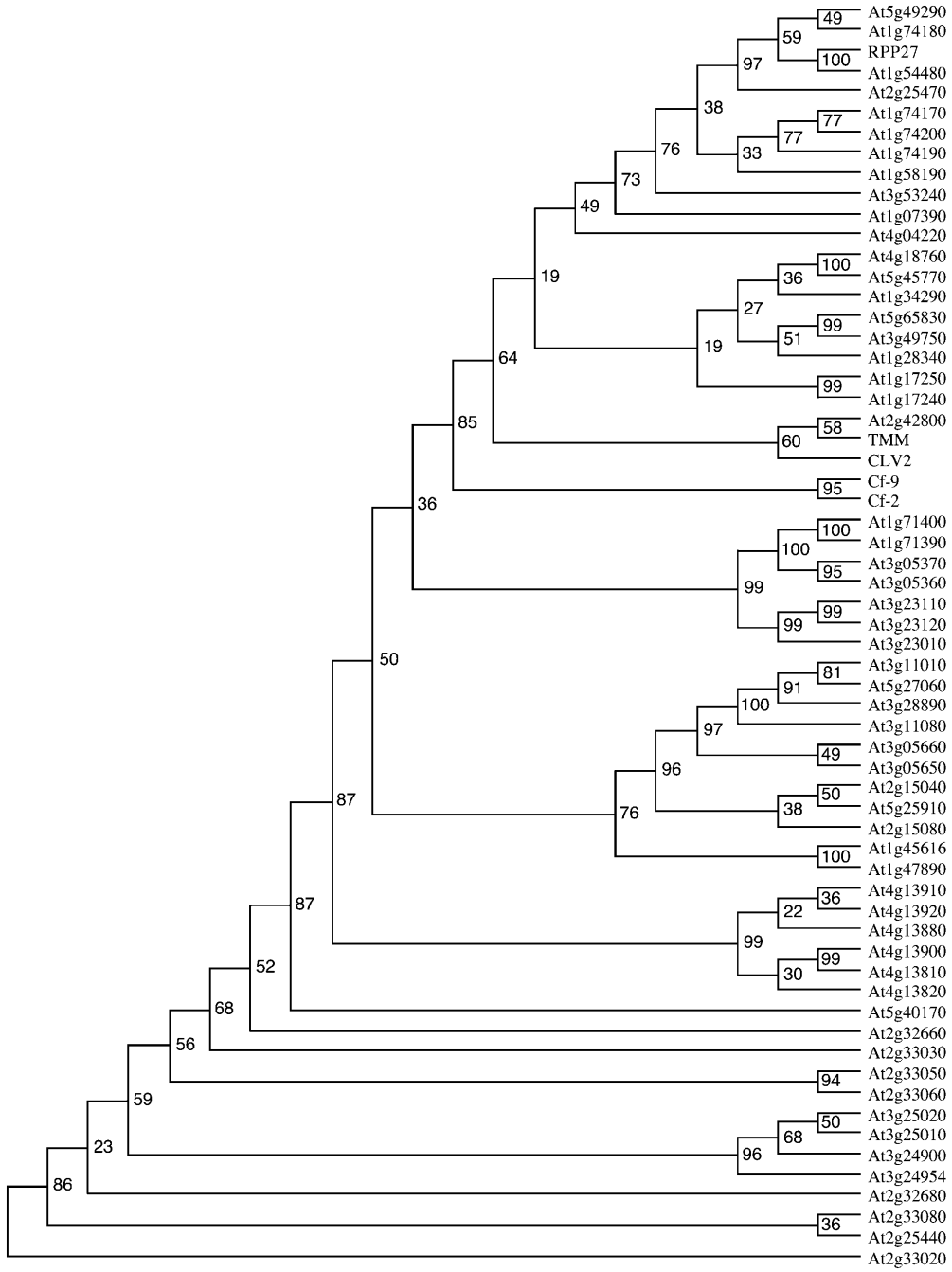

Figure 5. Phylogenetic tree of the RLP family in Arabidopsis. Amino acid sequences of 59 RLPs from Arabidopsis, as well as *Cf-2* and *Cf-9*, were aligned with MAFFT. The tree was generated from a truncated alignment consisting of the conserved C3-F domains. A total of 100 bootstrap replicates of this alignment were made. PHYLIP's Neighbor program was used to build the trees, and the Consense program generated the consensus tree and bootstrap values. Other bootstrapped trees (not shown) were built with parsimony and maximum likelihood methods from the full and truncated alignments. All trees were similar to this one, in that certain subfamilies appeared in every tree with high bootstrap values. The joining of these subfamilies in the higher nodes was inconsistent and invariably gave low bootstrap values. Note that At1g54480 is the Col-0 allele of *RPP27*.

signal peptide (M1-S20), followed by Leu-rich (Q21-S143) and LRR domains (I144-G946), an acidic region (D973-L997), a transmembrane domain (A998-F1020), and a short cytoplasmic tail (D1021-P1044). Analysis of the family of Arabidopsis RLPs reveals a conserved region of approximately 150 amino acids immediately preceding the transmembrane domain (Fig. 4). The number of LRR motifs is extremely variable across Arabidopsis RLPs; some members of the family have as few as four apparent repeats, while others have as

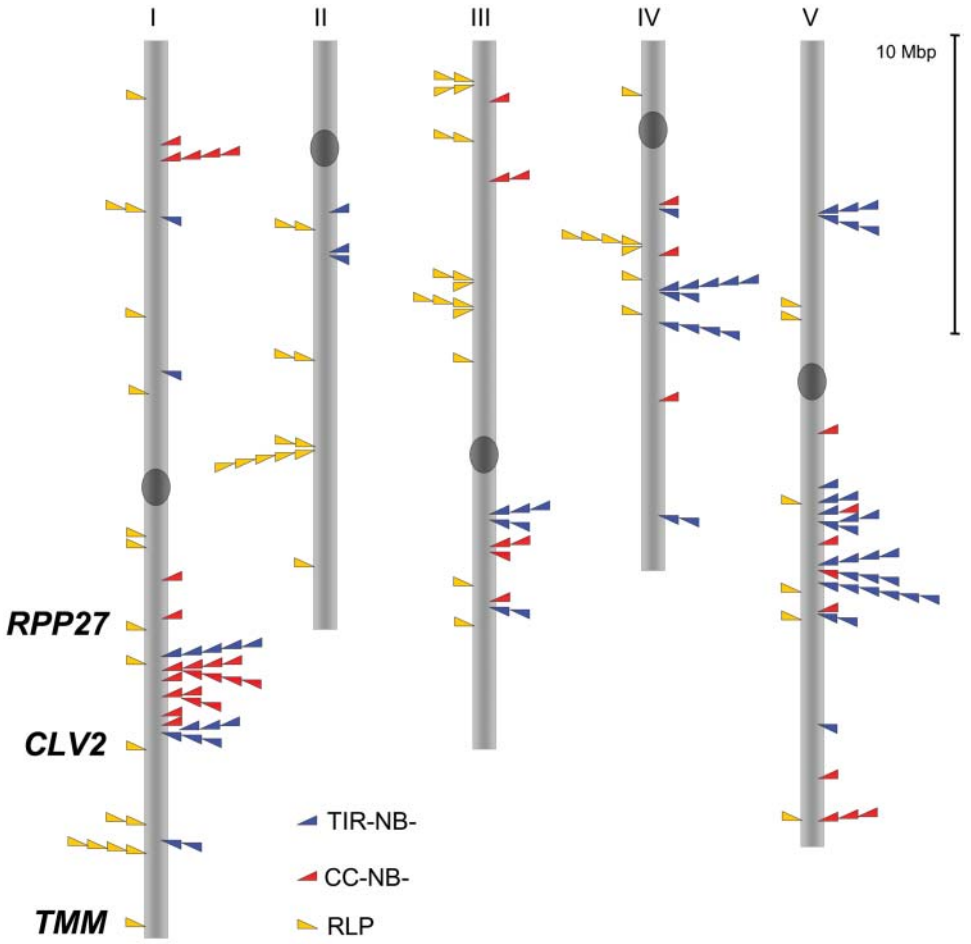

Figure 6. Distribution of RLPs in Arabidopsis relative to two subclasses of NB-LRR genes.

many as 49 repeats. The LRR motif itself varies in form across family members and at positions; some are extremely hard to detect using sequence-based methods. A hypervariable region of approximately 50 to 75 amino acids is found nested between detectable LRR motifs in many RLPs; the precise role played by this region is not known. In *RPP27*, this hypervariable region is found between residues 789 and 855.

The majority of the *RPP27* protein comprises extracellular LRR. However, there is no signal transduction domain, suggesting that additional proteins are required to facilitate the transmission of an *Avr*-induced conformational change from the extracytoplasmic to cytoplasmic domain and subsequently to activate the defense response. A possible function for the *RPP27* protein can be proposed based on the hypothesized model of the *CLV* family in Arabidopsis. According to this model, the RLP encoded by *CLV2* (Jeong et al., 1999) and an extracellular LRR RLK encoded by *CLV1* (Clark et al., 1997) form heterodimers and potentially act as a receptor for *CLV3*, a small secreted ligand (Fletcher et al., 1999), to activate the signal transduction cascade with the involvement of the kinase-associated protein phosphatase KAPP (Stone et al., 1998). Involvement of heterodimerization has also been suggested for other receptor-mediated signaling pathways in plants. For example, the *S* locus receptor kinase (*SRK*; Stein et al., 1996), and the *S* locus glycoprotein (*SLG*; Stein et al., 1991) genes are required for self-incompatibility in Brassica. It has been proposed that the S domains of *SRK* and *SLG* form a heterodimer after binding the pollen-derived ligand and activate the signaling pathway through the kinase domain of *SRK* (Stein et al., 1996).

Similar modes of action for some of the *R* genes are also proposed. The rice gene *Xa21* encodes an RLK and confers resistance to the bacterial pathogen *X. oryzae* pv *oryzae* (Song et al., 1995). However, *Xa21D* (Wang et al., 1998), a natural variant of *Xa21*, lacks the kinase and the membrane domain and still confers resistance to the same isolates of the pathogen. Wang et al. (1998) hypothesized that *Xa21D* forms a heterodimer with an endogenous RLK and activates the signaling cascade. Similar mechanisms for cytoplasmic signaling have been investigated for the *Cf-9* protein (Rivas et al., 2002) and proposed for the tomato *Ve* genes (Kawchuk et al., 2001).

To date, information on the RLP-mediated defense responses have come mainly from the studies with the *Cf* genes. Early *Cf*-mediated responses, including the

production of active oxygen species (Piedras et al., 1998), involvements of mitogen-activated protein kinases (Romeis et al., 1999), calcium-dependent protein kinases (Romeis et al., 2002), and *ACRE* genes (Durrant et al., 2000), have been reported. In addition, *Rcr3*, a secreted Cys protease (Krüger et al., 2002), has been cloned and shown to be a positive regulator of *Cf-2*-dependent resistance and autonecrosis. However, studying the signaling components of tomato *Cf* genes has been hindered by the lack of large-scale mutagenesis and microarray analysis because of its large genome size. We have reported previously (Tör et al., 2002) that *RPP27*-mediated resistance functions independently of *AtSGT1b*. With the identification of *RPP27*, we can take advantage of techniques and tools developed for Arabidopsis to understand the mechanism of the RLP-mediated resistance. As a complementary approach, we are conducting large-scale mutant screens to identify genes that are involved in *RPP27*-mediated resistance.

Inoculation of *RPP27* transgenic Col-*rpp7.1* plants with different L*er*-0 incompatible isolates of downy mildew showed that *RPP27* confers full resistance to four isolates and partial resistance to Emco5 (Table I). Results with the isolate Noco2 are intriguing because this isolate was used to clone *RPP5* from L*er*-0, and there was no indication from genetic analysis for an additional *R*-gene specificity on chromosome 1 (Parker et al., 1997). There may be two possible explanations for this finding. First, although the *RPP27* gene was cloned by PCR using a proofreading enzyme, a PCR error may have been introduced, creating a new specificity. However, we found no differences between sequence of the PCR insert and the corresponding genomic region from the L*er*-0. In addition, sequencing the cDNA clones and RACE products revealed no sequence difference. Alternatively, a second gene in Col-0 that is tightly linked in repulsion to the *RPP27* gene in L*er*-0 may be playing a role in conferring resistance to Noco2. This second gene (designated *RPP29*) is currently being investigated.

In a plant-pathogen interaction, the products of *R* genes recognize the effector molecules of the pathogen either directly (Bryan et al., 2000) or through an interacting partner (Mackey et al., 2002) and trigger the downstream signaling pathways. In the case of *RPP27*, it is tempting to speculate that recognition occurs in the intercellular space through the participation of the LRR region of *RPP27* directly binding the effector molecules of the pathogen. Rethage et al. (2000) reported that intercellular wash fluids from infected Arabidopsis leaves have race-specific elicitor activity, indicating that effector molecules may be secreted from Peronospora into the intercellular space. However, they have yet to identify a peptide with potential avirulence activity. This method (De Wit and Spikman, 1982) has been successfully used to study tomato/Cladosporium interaction and to clone *Avr* genes from *C. fulvum* (Van Kan et al., 1991; Van Den Ackerveken et al., 1992). However, binding studies with *AVR9* peptide suggest that the *Cf-9* protein is not the primary receptor of *AVR9* (Kooman-Gersmann et al., 1996), and no *AVR9*/*Cf-9* affinity could be detected (Luderer et al., 2001). It is important to note here that although *C. fulvum* is a biotrophic pathogen of tomato, it grows exclusively in the intercellular space and it does not produce haustoria. By contrast, downy mildew produces haustoria into the cells of the host plant (Fig. 1), and *RPP27*-mediated resistance appears to be posthaustorial. Therefore, it may be that the recognition between *RPP27* protein and the corresponding A*vr* gene product occurs in the extrahaustorial matrix, the sealed area between the host and the haustorial cytoplasm (Manners and Gay, 1983). Alternatively, this haustorial parasite may also secrete proteins along its hyphae in intercellular spaces of the host similar to *C. fulvum.*

Downy mildew genes that elicit a defense response in Arabidopsis have been described as *Arabidopsis thaliana-recognized* (*ATR*) avirulence determinants, including the genetic identification of *ATR* loci that match six known *RPP* genes (Gunn et al., 2002). Although, to date, there is no cloned *ATR* gene from Peronospora, Rehmany et al. (2002) have reported fine-scale mapping of the *ATR1Nd* avirulence gene using an F_2 mapping population created between Maks9 and Emoy2 isolates. A similar approach has been initiated to identify *ATR27*.

MATERIALS AND METHODS

Plant Lines

Arabidopsis Col-0 and L*er*-0 were used in this study. The mutants Col-*sgt1b* and Col-*rpp7.1* were described elsewhere (Tör et al., 2002). All seeds were vernalized at 4°C for 3 to 4 d before germination. Growth conditions for the plants were as described previously (Holub et al., 1994).

Growth of Downy Mildew Isolates

All isolates of downy mildew (*Peronospora parasitica*) were maintained on Wassilewskija-*eds1* (Parker et al., 1996). Preparation of inoculum for experiments and the assessment of sporulation were as described (Tör et al., 2002).

Light Microscopy

Seedlings of infected and noninoculated controls were stained with DAB to detect H_2O_2 as described previously (Thordal-Christensen et al., 1997). To visualize development of Peronospora and microscopic lesions, cotyledons were stained with a solution of phenol, lactic acid, glycerol, and water (1:1:1:1) supplemented with 1 mg/mL of trypan blue according to a previously described method (Koch and Slusarenko, 1990).

Map-Based Cloning of *RPP27*

The linkage of *RPP27* locus to the molecular marker g4026 on chromosome 1 was described previously (Tör et al., 2002). In addition to 310 Hiks1 susceptible F_2 Col-*edm1* X L*er*-0 families used for cloning *AtSGT1b* (Tör et al., 2002), an additional 100 Hiks1 susceptible F_2 families were identified from the same cross and included for fine mapping of *RPP27*. Several PCR-based markers from either side of g4026 were generated using the sequences of BAC clones and information from the Cereon Genomics SNP/INDEL database (Cambridge, MA). Initially, *RPP27* was mapped to an interval spanned by NGA280 and the F12M16 (generated from the BAC clone F12M16). Then, the locus was fine

mapped between the markers AC1 and AC2. AC1 was generated using primers 5′CCCAATTAAAAGGCCAATCA-3′ and 5′TCGTTTCGGAACTGTACATTA-3′, and the polymorphism between Col-0 and L*er*-0 was detected by the presence of a deletion of 26 bp. AC2 was amplified with the primers 5′-CGGTCCAGGTCGATTTTACA-3′ and 5′-CGCCATTGCAATAAGCATTT-3′. The product was cleaved with *Mbo*II to reveal the polymorphism. Four overlapping BAC clones (T18A20, F15I1, F20D21, and T22H22) span the *RPP27* interval. The marker CFL was generated from the sequence information of the *Cf*-like gene on the BAC clone F20D21 using primers 5′-TTGTAATAAGAGTTTGCAGC-3′ and 5′-TCATGCACTATTGTAGCAGG-3′, and the polymorphism between Col-0 and L*er*-0 was detected by the presence of a 68-bp deletion. The RPP27 locus cosegregated with the marker CFL. The *Cf*-like gene on the BAC clone F20D21 was targeted for cloning. The candidate gene was amplified from L*er*-0 with the primers 5′-TCGCCTTTTTATTGGCGTGTCGCT-3′ and 5′-TCAAACGGGTTTAGTTGTGGTCTCAAA-3′ using a proofreading enzyme, Elongase (Invitrogen, Glasgow, UK), and blunt-end cloned into *Sma*I digested binary vector pCAMBIA3300 (http://www.cambia.org), which carries the *BAR* gene. The construct was designated as MT27 and used for transformation.

Agrobacterium-Mediated Transformation

The construct MT27 was electroporated into *Escherichia coli* strain DH10B, and positive clones were identified by PCR and sequencing. The construct was then introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation, and the Col-*rpp7.1* plants were transformed by the floral dip method (Clough and Bent, 1998). Transformants were selected by spraying 7-d-old plants grown in soil with 0.04% Basta (AgrEvo, Norfolk, UK). Selected plants were self-pollinated to produce T_2 seeds and tested with Hiks1 isolate of Peronospora. Homozygous T_3 plants were then obtained and used for the subsequent experiments.

RACE and Sequence Analysis

Total RNA was isolated from infected and noninfected cotyledons and flowers using the RNeasy plant mini kit (Qiagen USA, Valencia, CA) according to the manufacturer's instructions. RT-PCR was performed as described (Tör et al., 2002) using the *RPP27* specific primers 5′-TCACCGGCTTCTTCTCATTTGATCC-3′ and 5′-TGTTGGACATGTCAAGAACTGATAACG-3′. RACE was performed with GeneRacer kit (Invitrogen) with the modification of the manufacturer's instruction. Using the GeneRacer Kit (Invitrogen), 3′ RACE PCR was performed, and nested PCR was carried out with two gene-specific primers (GSPs): GSP1, 5′-ACGGGTGATATTCCAAGCTGGATGTCTAA-3′, and GSP2, 5′-TCCGTTCAAGTTTTACGAATCCACATTTG-3′, against the GeneRacer 3′ primer and GeneRacer 3′ nested primer, respectively. For 5′ RACE PCR, first strand cDNA was synthesized with a GSP, 5′-CACAATGGTACGTAAAAACTGAAGTGAAAT-3′, using ThermoScript RT-PCR kit (Invitrogen), for 90 min at 65°C and then for 5 min at 85°C, followed by RNase H treatment for 20 min at 37°C. Nested PCR was then performed with two GSPs, first GSP1, 5′-GGCAACATACTTTTGACTGAGGCGATGG-3′, then GSP2, 5′-CCACCAACACGTGACGGTAAGGATCCAG-3′, against the GeneRacer 5′ primer and GeneRacer 5′ nested primer, respectively. A touchdown PCR regimen 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 the Advantage 2 PCR Enzyme System (BD Biosciences Clontech, Oxford). The PCR products were analyzed by gel electrophoresis and cloned into pCR4-TOPO vector (Invitrogen).

For all sequencing, both DNA strands were sequenced using BigDye termination kit (Applied Biosystems, Foster City, CA) and separated on ABI 377 sequencer. Cloned products were sequenced using the universal M13 primers.

Bioinformatics

Arabidopsis databases TIGR (http://www.tigr.org/) and MIPS (http://mips.gsf.de/) were used for the initial annotation of the *RPP27* region. Bioinformatics software used to refine predictions of DNA and amino acid sequences included NIX and PIX (http://hgmp.mrc.ac.uk/). Structure prediction of *RPP27* was achieved using several Web servers, including Phylofacts (https://phylogenomics.berkeley.edu/phylofacts/), InterPro (http://www.ebi.ac.uk/), SMART (http://smart.embl-heidelberg.de/), and PFAM (http://pfam.wustl.edu/). Signal peptide and transmembrane domain prediction used the SignalP (http://www.cbs.dtu.dk/services/SignalP-2.0/) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) Web servers. The RLP family HMM was created and scored with custom software; other HMMs constructed for these analyses used the Sequence Alignment and Modeling software from University of California Santa Cruz (http://www.cse.ucsc.edu/research/compbio/sam.html). Multiple sequence alignments were constructed using the MAFFT software (Katoh et al., 2002). Phylogenetic trees were constructed using the neighbor-joining and parsimony algorithms available within the PHYLIP package (http://evolution.genetics.washington.edu/phylip.html), as well as the protML maximum likelihood algorithm (Adachi and Hasegawa, 1996). Consensus trees were derived using PHYLIP's consense software. Primer designs, in silico digests, and comparison of genomic and full-length cDNA sequences of *RPP27* were performed using VectorNTI (InforMax, Oxford).

The accession number of the BAC F20D21 is AC005287. The accession numbers for the *RPP27* genomic and the cDNA sequences are AJ585978 and AJ585979, respectively. The accession numbers for *CLV2*, *TMM*, *Cf-9*, and *Cf-2* are NP_17617, Q9SSD1, CAA05274, and T10504, respectively.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AC005287, AJ585978, AJ585979, NP_17617, Q9SSD1, CAA05274, and T10504.

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RETRACTION

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Tör M., Brown D., Cooper A., Woods-Tör A., Sjölander K., Jones J.D.G., and Holub E.B. Arabidopsis Downy Mildew Resistance Gene *RPP27* Encodes a Receptor-Like Protein Similar to *CLAVATA2* and Tomato *Cf-9*.

The authors wish to communicate the following retraction. This article reported that the allele of gene At1g54480 from Landsberg *erecta* (L*er*)-0 encodes a receptor-like protein that confers resistance to Hiks1 and at least four L*er*-0 avirulent/Columbia (Col)-0 virulent isolates of downy mildew (*Peronospora parasitica*) pathogen in an *rpp7.1* mutant background of Col-*gl1*. Since the publication of the article, the authors have been unable to repeat the results in the original Col-*rpp7.1* background as well as in other Col-*rpp7* mutants, including Col-*rpp7.6*, Col-*rpp7.7*, Col-*rpp7.8*, Col-*rpp7.9*, Col-*rpp7.10*, and Col-*rpp7.11*. The observed disease resistance in the putative transformants was likely due to seed contamination of the plant lines used for transformation. For this reason, and because the authors are no longer confident in the data in Figure 2 and Table I, they hereby retract their *Plant Physiology* article. Figure 1 showing the map location of the *RPP27* locus (for *SGT1b*-independent Hiks1 resistance) is not in question. However, the gene within this mapping interval from L*er*-0 that confers *RPP27* function has yet to be determined. The authors deeply regret this error and sincerely apologize for the inconvenience and confusion this mistake caused *Plant Physiology* and its readership.