

***Arabidopsis* RPP4 is a member of the RPP5 multigene family of TIR-NB-LRR genes and confers downy mildew resistance through multiple signalling components**

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Summary

In *Arabidopsis*, *RPP4* confers resistance to *Peronospora parasitica* (*P.p.*) races Emoy2 and Emwa1 (downy mildew). We identified *RPP4* in Col-0 as a member of the clustered *RPP5* multigene family encoding nucleotide-binding leucine-rich repeat proteins with Toll/interleukin-1 receptor domains. *RPP4* is the orthologue of *RPP5* which, in addition to recognizing *P.p.* race Noco2, also mediates resistance to Emoy2 and Emwa1. Most differences between *RPP4* and *RPP5* occur in residues that constitute the TIR domain and in LRR residues that are predicted to confer recognition specificity. *RPP4* requires the action of at least 12 defence components, including *DTH9*, *EDS1*, *PAD4*, *PAL*, *PBS2*, *PBS3*, *SID1*, *SID2* and salicylic acid. The *ndr1*, *npr1* and *rps5-1* mutations partially compromise *RPP4* function in cotyledons but not in true leaves. The identification of *RPP4* as a TIR-NB-LRR protein, coupled with its dependence on certain signalling components in true leaves, is consistent with the hypothesis that distinct NB-LRR protein classes differentially signal through *EDS1* and *NDR1*. Our results suggest that *RPP4*-mediated resistance is developmentally regulated and that in cotyledons there is cross-talk between *EDS1* and *NDR1* signalling and processes regulating systemic acquired resistance.

Keywords: *Arabidopsis*, *Peronospora parasitica*, *RPP4*, TIR-NB-LRR, defence signalling pathway.

Introduction

Plant resistance to biotrophic pathogens is conferred by highly specific resistance (*R*) genes that elicit effective defence responses on perception of pathogen *Avr* genes (Feys and Parker, 2000). All characterized *Arabidopsis thaliana* *R* genes encode nucleotide-binding (NB) sites and leucine-rich repeat (LRR) domains (Parker *et al.*, 2000). Approximately 130 genes encoding NB-LRR proteins are present in the *Arabidopsis* genome, and these can be grouped in two main classes based on their N-terminal domains (Meyers *et al.*, 1999). The CC-NB-LRR is a broad class of NB-LRR proteins that contains a putative heptad leucine-zipper or coiled-coil (CC) motif. The TIR-NB-LRR class shows similarity to the cytoplasmic effector portion of the *Drosophila* Toll and human interleukin-1 transmembrane receptors (TIR). Similarities between NB-LRR proteins and activators of apoptosis in animal cells

reinforce the notion that the TIR and CC domains may play a role in activation of downstream pathways (Van der Biezen and Jones, 1998a; Van der Biezen and Jones, 1998b). LRR domains have been implicated in the perception of pathogen *Avr* products (Botella *et al.*, 1998; Ellis *et al.*, 1999; Jia *et al.*, 2000; Parniske *et al.*, 1997; Thomas *et al.*, 1997).

In *Arabidopsis*, members of both NB-LRR classes confer resistance to the oomycete *Peronospora parasitica* and the bacterium *Pseudomonas syringae*, whereas one member of the CC-NB-LRR class also confers viral resistance (Bittner-Eddy *et al.*, 2000; Cooley *et al.*, 2000; Parker *et al.*, 2000). The apparent lack of a correlation between the predicted modular NB-LRR structures and recognition of certain pathogen types is consistent with the idea that the TIR and CC portions are involved in activation of defence

pathways, rather than in pathogen perception (Aarts *et al.*, 1998). However, in the flax *L* alleles both the LRR and TIR domains play a role in determining pathogen specificity (Ellis *et al.*, 1999; Luck *et al.*, 2000). Mutational analyses in *Arabidopsis* have identified several genes required for either of the two NB-LRR protein classes, e.g. *enhanced disease susceptibility 1 (eds1)*; Aarts *et al.*, 1998); *non-race-specific disease resistance 1 (ndr1)*; Century *et al.*, 1995); and *avrPphB susceptible 2 (pbs2)*; Warren *et al.*, 1999). The results of these studies suggest that TIR-NB-LRR proteins require *EDS1*, whereas CC-NB-LRR proteins require *NDR1* and *PBS2* for function. Thus in *Arabidopsis* at least two apparently mutually exclusive parallel defence-signalling pathways are differentially employed by NB-LRR proteins that are distinguished by their TIR or CC domains (Aarts *et al.*, 1998; Warren *et al.*, 1999). *PBS3* appears to be involved in both pathways, and possibly establishes a convergence point (Warren *et al.*, 1999). A notable exception is the CC-NB-LRR protein RPP8 for resistance to *P. parasitica* that exhibits little or no requirement for any of the tested components (Aarts *et al.*, 1998; McDowell *et al.*, 1998, 2000).

In *Arabidopsis* landrace Columbia (Col), *RPP4* confers resistance to *P. parasitica* races Emoy2 and Emwa1 (Holub *et al.*, 1994). *RPP4*-mediated resistance has been shown to involve multiple defence-signalling components, including *DETACHMENT 9 (DTH9)*; Mayda *et al.*, 2000); *EDS1* (Aarts *et al.*, 1998); *NDR1* (Century *et al.*, 1995; Century *et al.*, 1997); *NON-EXPRESSOR OF PR GENES 1 (NPR1)*, also known as *NIM1* and *SAI1*; McDowell *et al.*, 2000); *PHYTOALEXIN DEFICIENT 4 (PAD4)*; Glazebrook *et al.*, 1997); *PBS2* and *PBS3* (Warren *et al.*, 1999); and *SALICYLIC ACID INDUCTION-DEFICIENT 1 and 2 (SID1 and SID2)*; Nawrath and Métraux, 1999). In addition, *RPP4* function is compromised by the *rps5-1* defective allele of the CC-NB-LRR-encoding gene *RPS5* for resistance to *P. syringae*, possibly through interference with a shared signalling partner (Warren *et al.*, 1998). Furthermore, *RPP4*-mediated resistance is diminished by *in vivo* inhibition of phenylalanine ammonium lyase (PAL) enzyme activity, the entry point of the phenylpropanoid pathway leading to salicylic acid synthesis (Mauch-Mani and Slusarenko, 1996). In line with this result is the observation that removal of salicylic acid in plants expressing the bacterial salicylate hydroxylase gene *NahG* (Gaffney *et al.*, 1993) also compromises *RPP4* function (Delaney *et al.*, 1994; McDowell *et al.*, 2000).

RPP4-mediated resistance differs from other race-specific resistances conferred by *RPP* and *RPS* genes, in that it is affected by mutations in many signalling components rather than only a subset (Feys and Parker, 2000). To characterize further the basis of resistance to *P. parasitica* Emoy2 and Emwa1, we isolated the *RPP4* gene. *RPP4* maps on chromosome 4 within a genetic interval that

includes *RPP5* homologues which we have characterized previously (Noël *et al.*, 1999). *RPP5* in Landsberg *erecta* (*Ler*) confers resistance to *P. parasitica* race Noco2 and is the founder member of the multigene family with diverged members in different landraces (Noël *et al.*, 1999; Parker *et al.*, 1997). *RPP5* and its homologues belong to the TIR-NB-LRR class, and have variable configurations and numbers of LRRs (Noël *et al.*, 1999). At the Col-0 haplotype, eight polymorphic *RPP5* homologues are clustered within ≈ 80 kb (Noël *et al.*, 1999), and although many members carry retro-element insertions or frameshift mutations, analysis of the codon usage indicated that most genes once were, or still are, functional (Noël *et al.*, 1999).

We describe the identification of *RPP4* in Col-0 as the orthologue of *RPP5* in *Ler*. Besides mediating resistance to *P. parasitica* Noco2, we show that *RPP5* also confers resistance to *P.p.* races Emoy2 and Emwa1, and thus accounts for the *Ler RPP4* specificity. Comparison between *RPP4* and *RPP5* identifies residues within the TIR and LRR domains that may be important for recognition specificity. Like *RPP5*, *RPP4* interacts in a yeast two-hybrid assay with an *Arabidopsis* RelA/SpoT homologue (Van der Biezen *et al.*, 2000). The identification of *RPP4* as a TIR-NB-LRR protein coupled with the involvement of *DTH9*, *EDS1*, *NDR1*, *NPR1*, *PAD4*, *PAL*, *PBS2*, *PBS3*, *rps5-1*, *SID1*, *SID2* and salicylic acid illustrates the complexity by which this class of NB-LRR proteins signals to activate defence.

Results

RPP5-ColA confers RPP4 function

For fine mapping of *RPP4*, we crossed Col-0 to the *P. parasitica* race Emwa1-susceptible landrace Wassilewskija (*Ws-0*). In the resulting F_2 population ($n = 1243$), 291 plants homozygous at the *Ws-0 rpp4* locus were identified by the full susceptibility of their first true leaves to *P.p.* Emwa1. The proportion of resistant versus susceptible individuals corresponds ($P = 0.05$) to a 3 : 1 ratio ($\chi^2 = 1.67$), confirming that *RPP4* is the only Col-0 locus conferring resistance to *P.p.* Emwa1. Plants heterozygous at *RPP4* were partially resistant, and the cotyledons allowed moderate to full asexual sporulation. This indicates that *RPP4* exhibits incomplete dominance. Susceptible individuals (*rpp4/rpp4*) were examined with molecular markers (see Experimental procedures). *RPP4* mapped between markers *VRN2* and *FCA8-2* and co-segregated with the *G4539* marker (Figure 1a), delimiting *RPP4* to a physical segment of maximally 304 kb (GenBank Z97342 and Z97343). This region includes eight *RPP5* homologues (Noël *et al.*, 1999) and 55 other predicted genes, none of which is an obvious *R* gene candidate (Bevan *et al.*, 1998). We therefore tested the possibility that one or more Col-0 *RPP5* homologues conferred resistance to *P.p.* races Emoy2 and Emwa1.

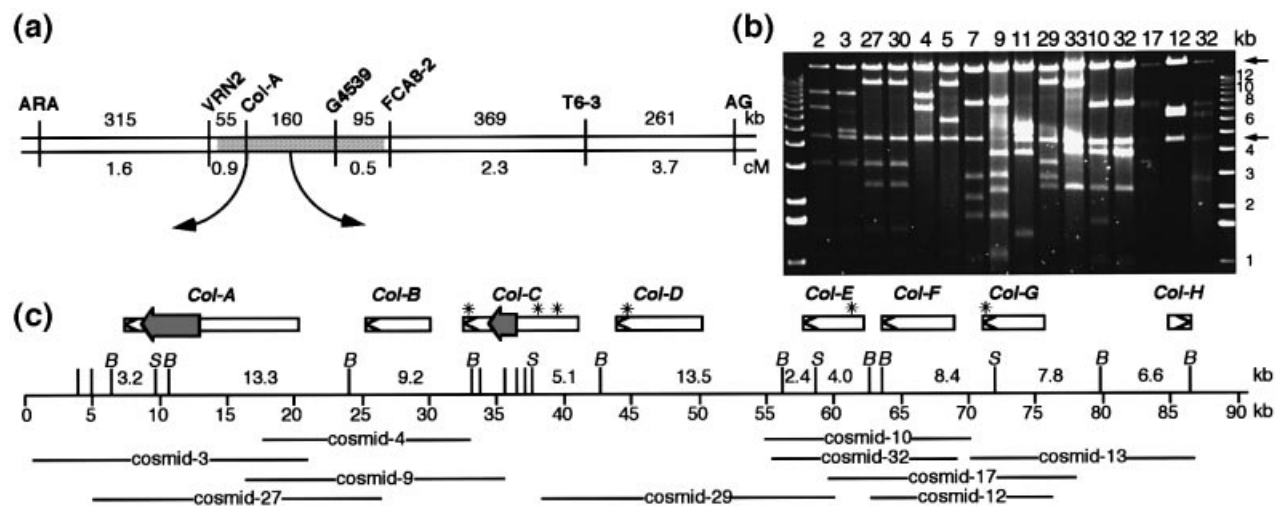


Figure 1. Mapping of *RPP4* at the Col-0 *RPP5* locus and identification of Col-0 *RPP5* homologues in binary cosmid vectors.

(a) *RPP4* maps within a short segment (grey area) on the long arm of chromosome 4 (left, centromeric; right, telomeric) defined by molecular markers (described in Experimental procedures) at intervals expressed in kilobases (kb, top) and centiMorgans (cM, below). The region indicated by the arrows contains eight *RPP5* homologues.

(b) Restriction mapping of cosmid inserts to identify specific Col-0 *RPP5* homologues. *Bam*HI and *Sal*I-digested cosmid DNA was separated through a 1% agarose-TAE gel, stained with ethidium bromide and photographed during exposure to UV light. Fragment sizes were inferred from co-migrating marker DNA at the borders of the gel, and are indicated in kilobases (kb). The arrows indicate 20 kb *Bam*HI/*Sal*I and 4.5 kb *Sal*I/*Sal*I cosmid vector fragments.

(c) Cosmid contig of the Col-0 *RPP5* haplotype (Noël *et al.*, 1999). The *RPP5* homologues (*Col-A* to *Col-H*) are shown as boxes with the direction of transcription indicated by arrowheads. Asterisks indicate the positions of the open reading frame-disrupting point mutations. Retro-element insertions are shown as grey arrowheads. The restriction map (B, *Bam*HI; S, *Sal*I) is shown below the *RPP5* homologues with sizes in kilobases (kb). The 10 cosmids (thick lines) were mapped on the genomic sequence by restriction analysis and DNA sequencing.

Ten binary cosmids were identified that collectively contained all six candidate Col-0 *RPP5* homologues (Figure 1b,c), and these were transformed into the *P.p.* Emoy2- and Emwa1-susceptible line CW-84 (see Experimental procedures). Self-progenies of the transgenic CW-84 lines (T_2) were then tested for complementation of resistance to *P.p.* Emoy2 and Emwa1. Only T_2 progenies with cosmid-3 or cosmid-27 segregated for resistance to these *P. parasitica* races, whereas progenies segregating for the other cosmids allowed profuse asexual sporulation, resembling non-transformed CW-84 controls (Figure 2; Table 1). T_3 progeny-testing by infection assays, PCR analysis, and kanamycin-resistance (transformation marker) assays showed that resistance to *P.p.* Emoy2 and Emwa1 co-segregated with cosmid-3 or cosmid-27. T_3 plants homozygous for the cosmid-3 or cosmid-27 inserts were all resistant to *P.p.* races Cala2 and Noco2, providing evidence for recognition specificity of the transgenes (Table 1). Examination of lactophenol-trypan blue-stained leaves infected with *P.p.* Emoy2 or Emwa1 showed that the resistance responses of transgenic CW-84 lines with cosmid-3 or cosmid-27 was similar to the resistance of Col-0 (Figure 2).

The complementing and overlapping cosmid-3 and cosmid-27 contain the *RPP5-ColA* gene (7673 bp) that carries a *Copia*-like retro-element insertion at its 3'-end

(5213 bp; Figure 1c; Noël *et al.*, 1999). Cosmid-3 has an insert size of 22.2 kb, and contains 895 bp 5' of the predicted ATG translation start codon of *RPP5-ColA*, and 8.5 kb 3' of the predicted stop codon. This region includes a predicted gene with weak similarity to *ubiquinol-cytochrome-c reductase (UCR)*. Cosmid-27 has an insert size of 23.0 kb, and contains 7.8 kb sequence 5' of *RPP5-ColA*, and 2.3 kb 3' of its stop codon (with only one-third of the *UCR* gene). From the complementation experiments and the 16.1 kb overlap between cosmid-3 and cosmid-27, we concluded that *RPP4* specificity is conferred by *RPP5-ColA*. Like *RPP5* in *Ler*, the *RPP5-ColA* homologue, hereafter referred to as *RPP4*, occupies the most centromeric position within the *RPP5* gene cluster in Col-0 (Figure 1c; Noël *et al.*, 1999).

Structure of *RPP4* and predicted amino acid sequence

RPP4 and *RPP5* are highly homologous and structurally very similar, including the position and number of introns (Figure 3a). The retro-element is inserted at the codon for amino acid residue 1113 and prevents expression of the last 130 codons and 3'-untranslated region (UTR); the first stop codon in the retro-element occurs after 30 codons (Figure 3b). The last exon of *RPP5*, encoding 26 amino acids and the 3'-UTR, was also found to be not required for function (Parker *et al.*, 1997). These portions do not encode

Table 1. Transgenic complementation of resistance to *P. parasitica*

CW-84 genotype	Number of lines tested ^a	<i>P. parasitica</i> races			
		Cala2	Emoy2	Emwa1	Noco2
Non-transformed control	1	S ^b	S	S	S
Cosmids 3/27	12 each	S	R	R	S
Cosmids 4/9/10/12/13/17/29/32	12 each	S	S	S	S
<i>RPP5</i>	7	S	R	R	R

^aBetween 30 and 60 T_2 individuals obtained by selfing of primary T_1 transformants were analysed in at least two separate experiments for segregation of resistance and the transgene.

^bR, resistant progenies (no asexual sporulation and no mycelium); S, susceptible progenies (full asexual sporulation and full mycelium).

LRRs and have no apparent homologies in the databases. Apart from *RPP4*, all other 17 *RPP5* homologues in Col-0 and *Ler* have an intron-1 that is nearly identical in size (126 bp) and sequence (Noël *et al.*, 1999). The unusually large intron-1 (3.2 kb) of *RPP4* is probably the result of an independent insertion event as the flanking regions are similar to those of *RPP5* and other homologues. Intron-1 of *RPP4* is A/T-rich (77%) and does not show similarity to other sequences in the databases. *RPP4* also contains an in-frame deletion in exon-6 of a 276 bp DNA fragment encoding four LRRs, which is a unique event within the *RPP5* family (Figure 3b; Noël *et al.*, 1999).

Comparison of the predicted amino acids shows highly related RPP4 and RPP5 proteins (74% amino acid identity/78% similarity; Figure 3b). The TIR domains (RPP4 residues 1–157) are most different (21% divergence), whereas the NB-ARC domains (RPP4 residues 158–516) display only 7% divergence (Figure 3b). RPP4 contains 17 LRRs which show 18% divergence with these of RPP5 (21 LRRs). Individual LRRs of the RPP5 family on average consist of 24 residues, of which five highly variable residues of the xxLxLxx motif (L = leucine, x = any amino acid) are predicted to be solvent-exposed and to specify pathogen recognition. The remaining residues are conserved and are predicted to serve a structural function (Noël *et al.*, 1999). RPP4 and RPP5 show only 12% divergence in their structural LRR residues (36 out of 306 residues) but display 40% divergence (34 out of 84 residues) in their solvent-exposed residues (Figure 3b). Previous codon-usage analysis in *RPP4* indicated divergent selection of solvent-exposed LRR residues (Noël *et al.*, 1999).

RPP4 and *RPP5* require multiple defence-component genes

In cotyledon assays, *RPP4* function has been shown to need various signalling components (Aarts *et al.*, 1998; Century *et al.*, 1995; Century *et al.*, 1997; Glazebrook *et al.*, 1997; McDowell *et al.*, 1998; Warren *et al.*, 1998; Warren *et al.*, 1999; Table 2). To examine the requirement for

RPP4-mediated resistance to *P.p.* races Emoy2 and Emwa1 in true leaves, we analysed the responses of seven Col-0 defence mutants and a Col-0 *NahG* transformant line (Table 2). The *DTH9* (Mayda *et al.*, 2000), *SID1* and *SID2* mutants (Nawrath and Métraux, 1999) were tested in other studies. The amount of hyphal growth in the *pbs2* and *pbs3* mutants was comparable to that of the genetically compatible interactions of *P.p.* Emwa1 with *Ws-0* or *P.p.* Noco2 with Col-0 (Figure 4a,g,h; Table 2). The *eds1-2* mutant plant supports even higher levels of *P. parasitica* growth (Figure 4c; Table 2) consistent with *eds1* conferring an 'enhanced disease susceptibility' phenotype (Parker *et al.*, 1996). Compared to *eds1-2* (Figure 4c) or to compatible interactions with wild-type plants (Figure 4a), significantly less asexual sporulation and lower amounts of mycelium were observed in the *pad4-1* mutant (Figure 4d; Table 2). Furthermore, in *pad4-1* *P. parasitica* hyphae were surrounded by mesophyll cells that retained the lactophenol-trypan blue stain (Figure 4b,g), and also fluoresced during exposure to ultra violet (UV) light (Figure 4f). Fluorescence is commonly observed in cells that have undergone a hypersensitive response (HR) and is probably due to the accumulation of phenolic compounds. The dead or dying plant cells accompanying the hyphae in *pad4-1* mutants may therefore be the result of a delayed HR that only partially prevents pathogen growth. *RPP4*-dependent trailing necrosis was also observed previously in *DTH9* (Mayda *et al.*, 2000), *SID1* and *SID2* mutants (Nawrath and Métraux, 1999).

A slight but quantitative increase of *P.p.* Emoy2 and/or Emwa1 asexual sporulation was previously observed in cotyledons of the Col-0 mutants *ndr1-1*, *npr1-1* and *rps5-1* (Century *et al.*, 1995; Century *et al.*, 1997; Glazebrook *et al.*, 1997; Holub *et al.*, 1994; McDowell *et al.*, 2000; Warren *et al.*, 1998). We confirmed these results by microscopic inspection of infected cotyledons. However, we found that in true leaves of these mutants, *P.p.* Emoy2 and Emwa1 resistance was not compromised. Asexual sporulation was not observed and mycelial growth was not detected in lactophenol-trypan blue-stained tissues (Figure 4i;

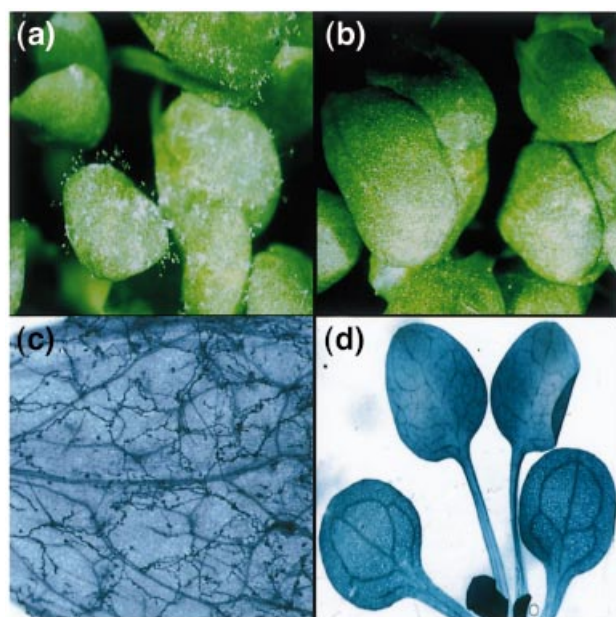


Figure 2. Cosmid-3 and cosmid-27 confer *RPP4* function. Complementation of the universally susceptible recipient CW-84 by cosmid-3 and cosmid-27 for resistance to *P. parasitica* races Emoy2 and Emwa1.

(a) Asexual sporulation on *P. parasitica* race Emoy2-infected true leaves of 2-week-old CW-84 seedlings.
 (b) Resistance to infection by *P. parasitica* Emoy2 of a typical CW-84 transformant containing a single-copy insert of cosmid-27.
 (c) Lactophenol-trypan blue staining of *P. parasitica* Emoy2 hyphae in true leaves of CW-84 photographed at 100 \times magnification.
 (d) Absence of hyphae in lactophenol-trypan blue-stained true leaves of a typical CW-84 transformant carrying cosmid-27.

Table 2). Hence the requirement by *RPP4* for the signalling components *EDS1*, *PAD4*, *PBS2* and *PBS3* is manifested in both cotyledon and true leaf tissues, while the effect of the *ndr1-1*, *npr1-1* and the *rps5-1* mutations is observed in cotyledons only. The differential requirement for signalling components in two different organs suggests that *RPP4*-mediated resistance may be developmentally or tissue-specifically regulated.

Salicylic acid is essential for the establishment of systemic acquired resistance (SAR; Delaney *et al.*, 1994; Gaffney *et al.*, 1993), and has been suggested to play a major role in *RPP4*-mediated resistance in true leaves (Mauch-Mani and Slusarenko, 1996). Moreover, the bacterial salicylate hydroxylase gene *NahG*, which converts salicylic acid to catechol (Gaffney *et al.*, 1993), compromised *RPP4* function in cotyledons (Delaney *et al.*, 1994; McDowell *et al.*, 2000) and in true leaves (Nawrath and Métraux, 1999; Table 2). In addition, the *sid1* and *sid2* mutants that are impaired in the pathway leading to salicylic acid biosynthesis are also required for *RPP4* function in true leaves (Nawrath and Métraux, 1999). We examined further the relationship of the *RPP4* pathway

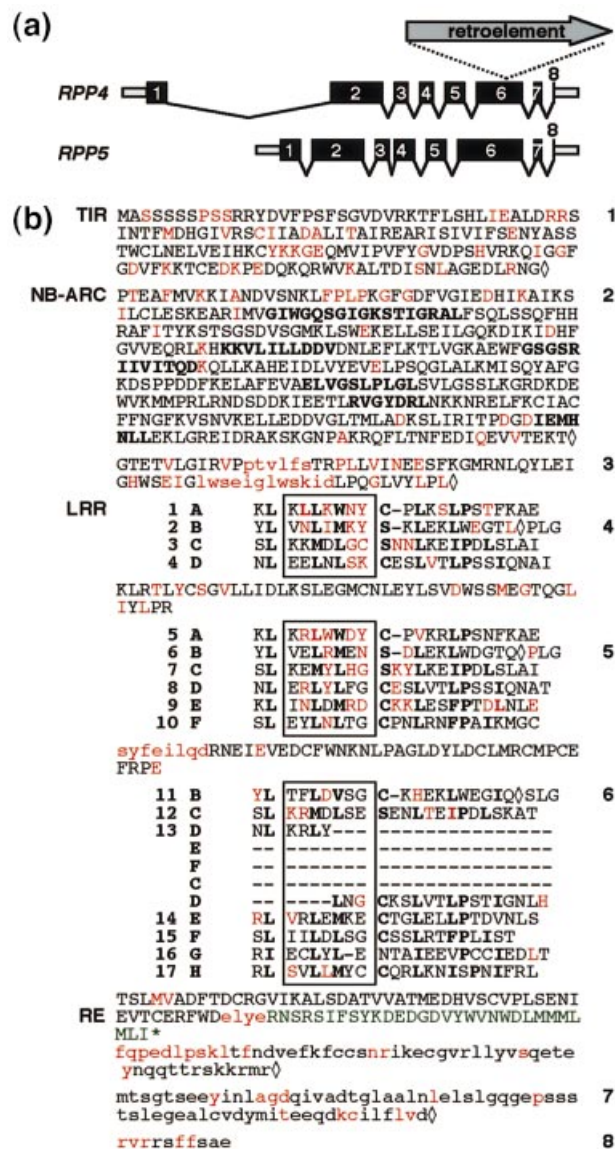


Figure 3. Comparison of the *RPP4* and *RPP5* genes and products. (a) *RPP4* and *RPP5* gene structures. The solid rectangles indicate coding regions (exons 1–8), narrow rectangles indicate 5' and 3' UTRs, and introns are shown by arrowheads. The *Copia*-like retro-element inserted at exon 6 of *RPP4* is shown as a grey arrow. Intron-1 of *RPP4* (3.2 kb) is markedly larger than that of *RPP5* (126 bp), and exon 6 of *RPP4* is shorter than that of *RPP5* because of an in-frame 276 bp deletion. (b) The *RPP4* protein and its predicted TIR, NB-ARC and LRR structures are shown as given by Parker *et al.* (1997) and Noël *et al.* (1999). Intron-exon boundaries are indicated by diamonds, and exon numbers are indicated at the right. The amino acid sequence of *RPP4* is shown and is compared to that of *RPP5*; black residues are conserved between *RPP4* and *RPP5*, and residues shown in red are different. The 30 predicted amino acid residues resulting from the retro-element (RE) insertion in exon-6 are shown in green; the first stop codon introduced by the retro-element is indicated by an asterisk. Residues shown in lower-case letters either are part of highly variable regions and cannot be aligned (exon-3) or, as a result of the retro-element insertion in exon-6, are predicted not to be expressed (exons 6–8). The deletion in the LRR region of *RPP4* relative to the corresponding region of *RPP5* is shown by dashes. Conserved motifs in the NB-ARC domain, and conserved hydrophobic residues within the LRRs, are shown in bold. Predicted solvent-exposed residues (x in the xxLxLxx motif) are shown in boxes.

defined by *EDS1*, *PAD4*, *PBS2* and *PBS3* in true leaves, with the *NPR1* pathway regulating activation of SAR in response to the salicylic acid analogue benzothiadiazole (BTH; Lawton *et al.*, 1996). Sporulation on BTH-treated Col-0 *eds1-2*, *pad4-1*, *pbs2* and *pbs3* mutants was not observed on true leaves, indicating that the SAR pathway downstream of BTH perception is still intact in these mutants (Table 2). As expected, foliar BTH application did not induce SAR in *npr1-1* plants (Table 2).

As *RPP4* and *RPP5* are highly homologous, we wished to compare their genetic requirements. The *eds1-2* mutation suppresses *RPP4* and *RPP5* function to a similar extent (Aarts *et al.*, 1998; data not shown). Also, *RPP5*-mediated

resistance to *P.p.* Noco2 in the *Ler pad4-2* mutant was affected in a similar way to *RPP4*-mediated resistance in a Col-0 *pad4-1* background, including the typical trailing necrosis (Feys *et al.*, 2001). Furthermore, like *RPP4*, *RPP5* function was not compromised by the *ndr1-1* and *npr1-1* mutations in true leaves (Aarts *et al.*, 1998; data not shown). The segregation of resistance to *P. p.* Noco2 in F_2 and F_3 progenies from a *Ler-RPP5* × Col-*pbs2* cross, and the loss of *RPP5* function in four selected F_3 plants homozygous for both *RPP5* and the *pbs2* mutation, showed that *PBS2* is fully required for *RPP5* function (data not shown). These results indicate that, in true leaves, *RPP4* and *RPP5* similarly require the *EDS1*, *PAD4*

Table 2. Mutations affecting *RPP4*-mediated resistance and systemic acquired resistance

Genotype	<i>P. parasitica</i> races Emoy2 and Emwa1			<i>P. parasitica</i> race Noco2	
	Cotyledons	True leaves		True leaves	
		Non-treated	BTH treated	Non-treated	BTH treated
Col-0 (<i>RPP4</i>)	+/- ^a	-	-	+++	-
Ws-0 (<i>rpp4</i>)	+++ ^b	+++ ^b	- ^b	-	-
Col/Ler <i>eds1-2</i> ^c	++++	++++	-	++++	-
Col-0 <i>ndr1-1</i>	+	-	-	+++	-
Col-0 <i>npr1-1</i>	+	-	-	+++	+++
Col-0 <i>pad4-1</i>	++	++	-	+++	-
Col-0 <i>pbs2</i>	+++	+++	-	+++	-
Col-0 <i>pbs3</i>	+++	+++	-	+++	-
Col-0 <i>rps5-1</i>	+	-	-	+++	-
Col-0 <i>NahG</i>	++	++	nd	++++ ^e	nd
Col-0 <i>sid1</i>	nd ^d	++ ^{be}	nd	++++ ^e	nd
Col-0 <i>sid2</i>	nd	++ ^{be}	nd	++++ ^e	nd
Col-0 <i>dth9</i>	nd	++ ^{bf}	nd	++++ ^f	nd

^a-, No mycelial growth and no sporulation; +, low mycelial growth and sporulation; ++, intermediate mycelial growth and sporulation; +++, full mycelial growth and sporulation comparable to compatible interactions with wild type plants; +++++, enhanced mycelial growth and sporulation.

^b*P. parasitica* race Emwa1 only.

^cSelected from a Col-*gl* × *Ler eds1-2* cross; homozygous at Col-*RPP4* and Col-*rpp8* (Aarts *et al.*, 1998).

^dnd, Not determined.

^eData from Nawrath and Métraux (1999).

^fData from Mayda *et al.* (2000).

Figure 4. *RPP4* function requires a multitude of signalling components.

True leaves of 2-week-old Col-0 wild-type and mutant seedlings were infected with *P. parasitica* and after 5 days stained with lactophenol-trypan blue.

(a) Col-0 wild-type control infected with the compatible *P.p.* race Noco2. Hyphae ramify abundantly through mesophyll tissue.

(b) Interacellular *P.p.* Noco2 hyphae with intracellular haustoria in Col-0 wild-type mesophyll cells (100× magnification).

(c) Col-0/*Ler eds1-2* mutant infected with *P.p.* Emoy2 shows enhanced susceptibility.

(d) Col-0 *pad4-1* mutant infected with *P.p.* Emoy2 shows partial resistance.

(e) *P.p.* Emoy2 hyphal ramification in the Col-0 *pad4-1* mutant induces death of surrounding (lactophenol-trypan blue-stained) mesophyll cells (100× magnification).

(f) Dead or dying mesophyll cells accompanying *P.p.* Emoy2 hyphae in the Col-0 *pad4-1* mutant fluoresce during exposure to UV light (100× magnification).

(g) Col-0 *pbs2* mutant infected with *P.p.* Emoy2 shows full susceptibility.

(h) Col-0 *pbs3* mutant infected with *P.p.* Emoy2 shows full susceptibility.

(i) Col-0 *ndr1-1* mutant infected with *P.p.* Emoy2 shows no effect on *RPP4* function.

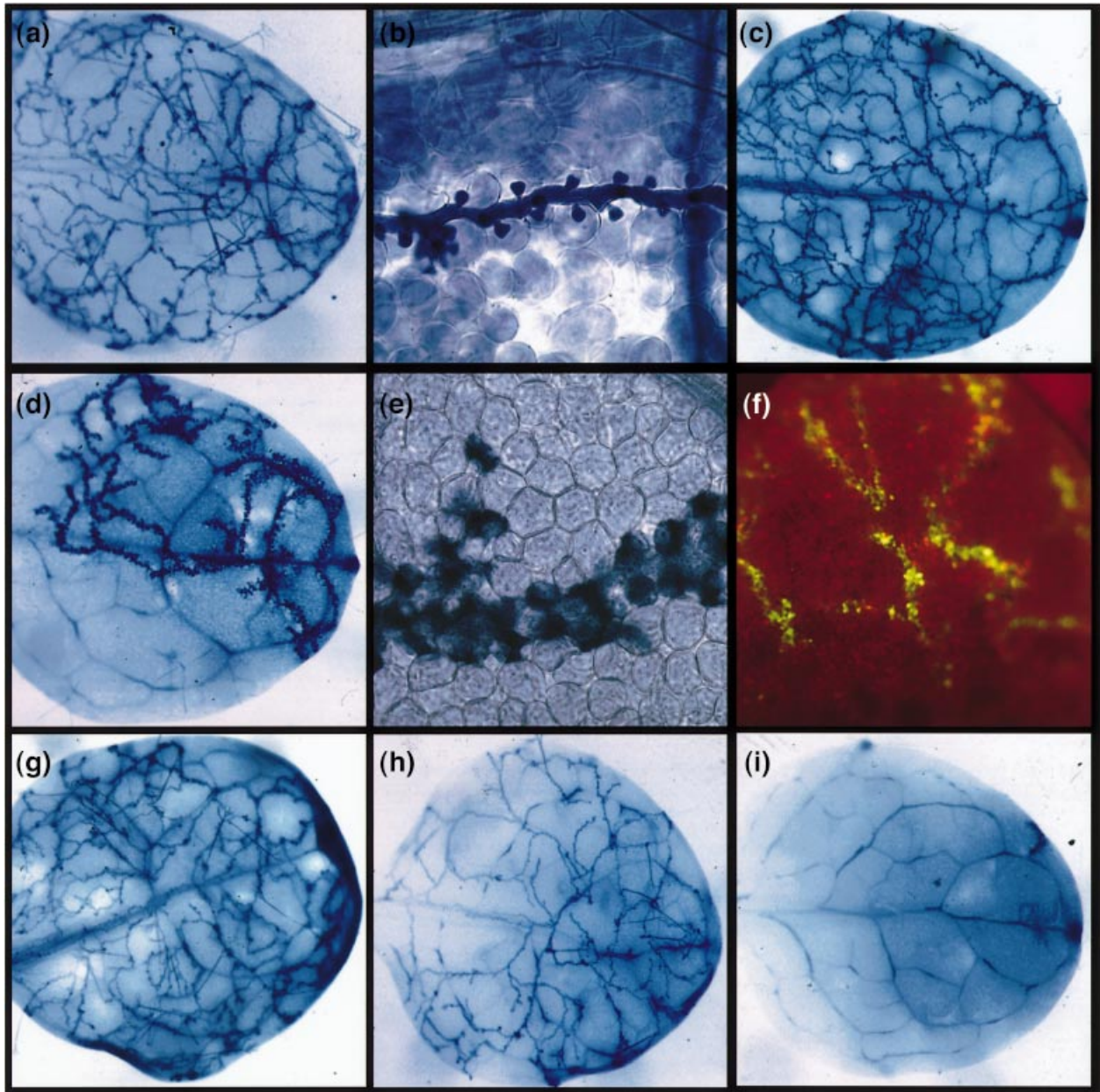


Figure 4. Legend on facing page.

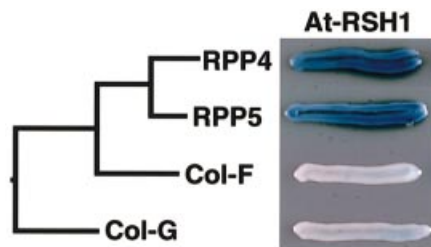


Figure 5. *RPP4* and *RPP5* interact in a yeast two-hybrid assay with the *Arabidopsis RelA/SpoT* homologue At-RSH1. A dendrogram of NB-ARC domains of *RPP4*, *RPP5* and the *RPP5* homologues *Col-F* and *Col-G* shows protein-sequence distance relationships (Noël *et al.*, 1999). The NB-ARC domain of *RPP5* was previously shown to interact in a yeast two-hybrid assays with *At-RSH1* (Van der Biezen *et al.*, 2000). Here we report that the closely related NB-ARC domain of *RPP4* also activates the *LacZ* (blue) and *LEU2* (not shown) reporters. The NB-ARC domains of the more distantly related homologues *Col-F* and *Col-G* do not activate the *LacZ* (white) and *LEU2* reporters.

and *PBS2* genes, but do not appear to require the *NDR1* and *NPR1* genes. The effects of the *pbs3* and *rps5-1* mutations on *RPP5* function were not tested.

RPP4 and RPP5 interact in yeast with an Arabidopsis RelA/SpoT homologue

We previously separated the NB-ARC domain of the *RPP5* family in an NB site (≈ 130 residues) and an ARC domain (≈ 180 residues; Noël *et al.*, 1999; Van der Biezen and Jones, 1998a). Phylogenetic grouping of all full-length *RPP5* family members showed that NB sites are nearly identical, while there are three different classes of ARC domains (Noël *et al.*, 1999). The ARC domain of *RPP4* belongs to the same class of that of *RPP5*. We reported that the full-length NB-ARC domain of *RPP5* is necessary and sufficient to interact in a yeast two-hybrid assay with a C-terminal hydrophilic domain (≈ 160 residues) of the *Arabidopsis* *RelA/SpoT* homologue, *At-RSH1* (Van der Biezen *et al.*, 2000). It is not known whether the interaction with *At-RSH1* is relevant for *RPP5* function. We show here that co-expression in yeast of *At-RSH1* with the NB-ARC domain of *RPP4* activates the two-hybrid reporter genes in a similar manner to that of *RPP5* (Figure 5). In contrast, the same domain of the two other *RPP5* family members, *Col-F* and *Col-G*, which belong to the two other ARC classes, did not interact with *At-RSH1* (Figure 5).

RPP4 specificity in Landsberg erecta is conferred by RPP5

In *Ler*, resistance to *P.p.* Emoy2 and Emwa1 co-segregated with the *RPP5* locus and has been denoted *RPP4* (Holub *et al.*, 1994). However, it was not known whether *Ler RPP4* is a distinct gene or an allele of *RPP5* conferring *P.p.* Emoy2 and Emwa1 recognition. DNA sequence and transcript analysis indicated that *RPP5* is the only functional gene in the *Ler RPP5* haplotype (Noël *et al.*, 1999). Therefore we tested whether *RPP5* itself confers resistance to *P.p.* Emoy2 and Emwa1, besides mediating resistance to *P.p.* Noco2. In this analysis we used a *P.p.* Noco2-sensitive *Ler* mutant that carries the defective *rpp5-2* allele identified in an ethyl methanesulfonate (EMS)-mutagenized population (Louise N. Frost and J.E.P., unpublished results). The presence of the *RPP8* gene in *Ler* for resistance to *P.p.* Emoy2 and Emwa1 (McDowell *et al.*, 1998) prevented testing directly whether the *rpp5-2* mutant had also lost resistance to *P.p.* Emoy2 and Emwa1 resistance. We therefore conducted a segregation analysis. In F_2 progeny (>1000 plants) from the wild-type *Ler* \times *Col-0* control cross, we found no individuals that were susceptible to *P.p.* Emoy2 or Emwa1 infection, confirming that *RPP4* in *Col-0* and the recognition of *P.p.* Emoy2 and Emwa1 in *Ler* map at the same locus (Table 3). However, in F_2 progeny from the mutant *Ler rpp5-2* \times *Col-0* cross, individuals

susceptible to *P.p.* Emoy2 and *P.p.* Emwa1 occurred in a ratio of 1 : 15 resistant plants, consistent with the segregation of two unlinked genes, *Col-0 RPP4* and *Ler RPP8* (Table 3). The concomitant loss of *P.p.* Noco2, Emoy2 and Emwa1 resistance in the single-mutant *rpp5-2* indicates that *RPP5* recognizes not only *P.p.* race Noco2, but also *P.p.* races Emoy2 and Emwa1.

To confirm that *RPP5* confers resistance to *P.p.* races Emoy2 and Emwa1, CW-84 plants were transformed with the *RPP5* transgene (Parker *et al.*, 1997). In T_2 and T_3 progenies from seven independently transformed plants, the *RPP5* transgene co-segregated with resistance to *P.p.* races Noco2, Emoy2 and Emwa1 (Table 1). These results strongly support the conclusion that the *RPP4* specificity for *P.p.* Emoy2 and Emwa1 recognition in *Ler* is conferred by *RPP5*.

Discussion

Intraspecific allelic divergence at a complex disease-resistance locus

We identified *RPP4* for resistance to *P. parasitica* races Emoy2 and Emwa1 in the landrace *Col-0* as an allele of *RPP5* which also confers resistance to *P.p.* Noco2 in *Ler* (Parker *et al.*, 1997). We previously analysed the *RPP5* haplotypes of the landraces *Col-0* and *Ler* that comprise small multigene families of eight and 10 members, respectively (Noël *et al.*, 1999). Evolution of the *RPP5* locus involved pronounced haplotype divergence in several landraces, which included point mutations, deletion and duplication events, gene conversions, and intergenic and intragenic recombinations. Fifteen out of all 18 *RPP5* family members are truncated or carry frameshift mutations, suggesting that most *RPP5* homologues are not functional. However, the solvent-exposed, presumably ligand-interacting LRR residues are highly variable among the *RPP5* family, and were predicted to specify pathogen recognition in the recent evolutionary past (Noël *et al.*, 1999). Furthermore, analysis of the ratio of non-synonymous over synonymous nucleotide substitutions in codons encoding the solvent-exposed LRR residues provided evidence of divergent selection (Noël *et al.*, 1999). The cloning of *RPP4* shows that at least one of the six intact or 3'-truncated *Col-0* genes, *RPP5-ColIA*, is functional.

The most distinguishing features of *RPP4* are the large insertion in intron-1 (3.2 kb), the insertion of a retro-element at the 3'-end, and an in-frame deletion of a 276 bp segment encoding four LRRs, which are all unique events within the *RPP5* family (Figure 3). The similar position of *RPP4* and *RPP5* in the gene cluster of the *Col-0* and *Ler* haplotypes, respectively, and their close sequence affiliation characterized by informative polymorphic sequences, indicate that *RPP4* and *RPP5* are true

Table 3. RPP4 Specificity in Landsberg *erecta* (Ler) is conferred by RPP5. Segregation of the defective *rpp5-2* allele shows that loss of *P. parasitica* Noco2 resistance is accompanied by loss of resistance to *P.p.* Emoy2 and Emwa1

<i>P. parasitica</i> race	<i>F</i> ₂ progeny from wild-type <i>Ler</i> × Col-0			<i>F</i> ₂ progeny from <i>Ler rpp5-2</i> × Col-0		
	Resistant: susceptible ^a	Ratio	χ^2 ^b	Resistant: susceptible ^a	Ratio	χ^2 ^b
Cala2	147 : 44	3 : 1	0.39	182 : 56	3 : 1	0.27
Emoy2	>1000 : 0	1 : 0	0	432 : 24	15 : 1	0.76
Emwa1	>1000 : 0	1 : 0	0	216 : 11	15 : 1	0.77
Emco5 ^c	97 : 26	3 : 1	0.97	71 : 24	3 : 1	0
Noco2	132 : 46	3 : 1	0.07	0 : >1000	0 : 1	0

^aResistant, no asexual sporulation; susceptible, full asexual sporulation; these are combined results of two independent replicates.

^bGoodness-of-fit (χ^2) significant at *P* = 0.05 with 1 degree of freedom is 3.84.

^c*P.p.* Emco5 is recognized by *Ler RPP8* only (McDowell *et al.*, 1998).

Table 4. Recognition specificities in three *Arabidopsis* landraces of five *P. parasitica* races used in this study

<i>P. parasitica</i> race	Recognition of <i>P. parasitica</i> (RPP) by <i>Arabidopsis</i> landrace			Deduced presence of <i>P. parasitica</i> Avr genes ^a						
	Col-0	Ler	Ws-0	AvrRPP1A	AvrRPP1B	AvrRPP1C	AvrRPP2	AvrRPP4	AvrRPP5	AvrRPP8
Cala2	RPP2	none	RPP1A	+	-	-	+	-	-	-
Emoy2	RPP4	RPP5, RPP8	RPP1A,B	+	+	-	-	+	+	+
Emwa1	RPP4	RPP5, RPP8	none	-	-	-	-	+	+	+
Emco5	none	RPP8	none	-	-	-	-	-	-	+
Noco2	none	RPP5	RPP1A,B,C	+	+	+	-	-	+	+

^a+, Avr gene present; -, Avr gene absent.

orthologues (Noël *et al.*, 1999). Allelic variation at loci that specify resistance to different races of the same fungal species is commonly observed, e.g. the flax *L* alleles against rust races, barley *Mla* alleles against powdery mildew races, and the tomato *Cf* alleles against leaf mould races. Recently, Bittner-Eddy *et al.* (2000) cloned two alleles of the single-copy *Arabidopsis RPP13* gene that encode CC-NB-LRR proteins recognizing different *P. parasitica* races. The *RPP4* and *RPP5* alleles encode distinct specificities, which reinforces the contention that they have evolved from a common ancestral gene through adaptive selection to different pathogen races (Noël *et al.*, 1999). In natural populations like *Arabidopsis*, selection-driven allelic variation at resistance loci generates spatial and genetic diversity ('balancing polymorphisms') that may reduce the selection pressure for pathogens adapting to virulence by frequency-dependent selection (e.g. Noël *et al.*, 1999).

Different recognition specificities of the RPP4 and RPP5 alleles

In *Ler*, two unlinked genes specify resistance to *P.p.* Emoy2 and Emwa1: the *RPP8* gene on chromosome 5, and a

second gene that is linked to the *RPP5* locus (Holub *et al.*, 1994; Tör *et al.*, 1994). Although the latter *Ler P.p.* Emoy2/Emwa1 specificity could not be separated by recombination from *RPP5*, the gene was tentatively named *Ler RPP4* by inference from the *RPP4* gene in Col-5 that maps at a similar position and also confers resistance to the same *P.p.* races (Holub *et al.*, 1994; Tör *et al.*, 1994). We show here that *Ler RPP4* is not a distinct gene, and that *RPP5* confers *P.p.* Noco2, Emoy2 and Emwa1 recognition. Therefore *RPP4* and *RPP5* have overlapping specificities, which is best explained to occur through recognition of distinct avirulence (*Avr*) determinants (Table 4). *P.p.* races Noco2, Emoy2 and Emwa1 presumably share the *Avr* factor that is recognized by *RPP5* (i.e. *AvrRPP5*), and in addition, *P.p.* Emoy2 and Emwa1 carry an *Avr* gene that is recognized by *RPP4* (i.e. *AvrRPP4*; Table 4). A similar scenario has been postulated for recognition of different *P. parasitica* Avr genes by three *RPP1* paralogues (Botella *et al.*, 1998; Table 4), and by two *RPP13* orthologues (Bittner-Eddy *et al.*, 2000).

Characterization of functional alleles for resistance to different pathogen races or species has identified LRR residues involved in the determination of recognition (Bittner-Eddy *et al.*, 2000; Botella *et al.*, 1998; Cooley *et al.*,

2000; Ellis *et al.*, 1999; Thomas *et al.*, 1997; Van der Vossen *et al.*, 2000). LRR domains presumably form surfaces for direct or indirect interactions with pathogen-derived ligands (Jia *et al.*, 2000; Jones and Jones, 1996). RPP4 and RPP5 differ mainly in residues encoding the TIR domain, predicted ligand-interacting LRR residues, and in LRR copy number. Hypervariability of ligand-interacting LRR residues, and differences in LRR copy number, could create surfaces for different ligand interactions, and hence potential for differential pathogen recognition. The TIR domain has also recently been implicated in pathogen recognition by comparing flax *L* alleles that differ only in their TIR portions (Luck *et al.*, 2000). This is consistent with a role of the TIR domain as effector activating downstream signalling components (O'Neill and Greene, 1998). Defence activation presumably results from both recognition and intramolecular interactions involving the LRR and TIR domains (Van der Biezen and Jones, 1998b). Differences between the TIR domains of RPP4 and RPP5 may also result from co-evolution with the diverging LRR domains.

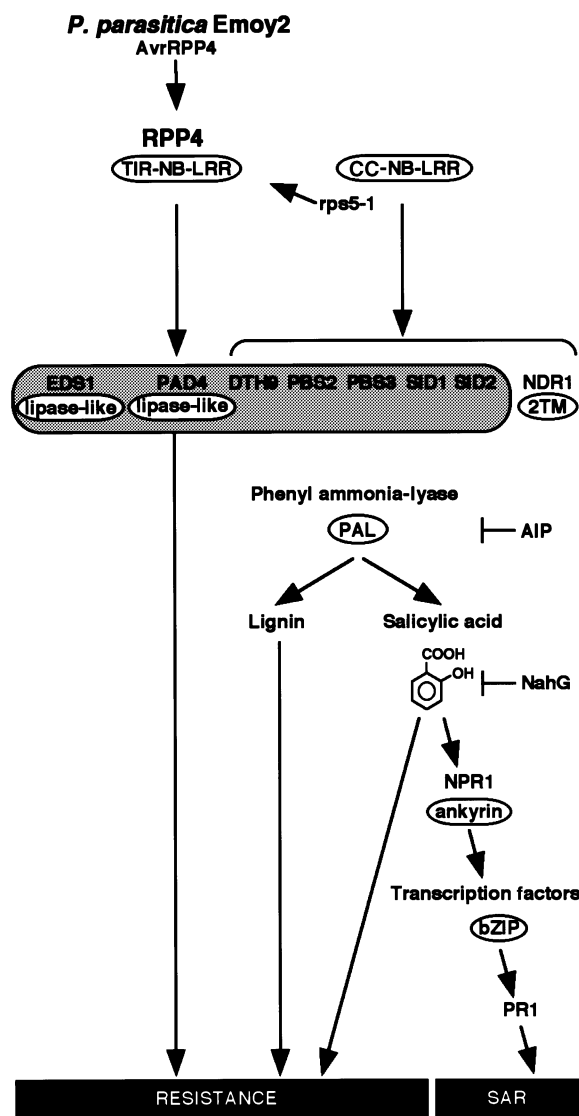
RPP4 and RPP5 function through multiple-signalling components

RPP4 signals through at least 12 defence components that were previously implicated in pathogen resistance (Table 2; Figure 6). *EDS1* was shown to be required by RPP1, RPP5 and RPS4 (resistance to *Pseudomonas syringae*) that all belong to the TIR-NB-LRR class (Aarts *et al.*, 1998). *NDR1* and *PBS2* were shown to be required for resistance to *P.*

syringae by RPS2, RPS5 and RPM1 which all belong to the CC-NB-LRR class (Aarts *et al.*, 1998; Warren *et al.*, 1999). *PBS3* was required for full function of both NB-LRR protein classes, i.e. RPS2, RPS5 and RPM1 and RPS4 (Warren *et al.*, 1999). The identification of RPP4 as a TIR-NB-LRR protein, and its requirement in true leaves for *EDS1* and *PBS3*, but not for *NDR1*, are in line with the differential utilization by the two NB-LRR classes of the two parallel resistance pathways defined by *EDS1* and *NDR1* that may converge at *PBS3* (reviewed by Feys and Parker, 2000). However, our results show that *PBS2* is required for signal transduction in both pathways (Warren *et al.*, 1999), and suggest a role of *PAD4* in the *EDS1* pathway (Figure 6). An *EDS1*-*PAD4* interaction has been inferred in other molecular and genetic studies (Feys *et al.*, 2001). The CC-NB-LRR protein RPS2 does not require *PAD4* (Zhou *et al.*, 1998), perhaps indicat-

Figure 6. RPP4-mediated defence signalling towards activation of resistance to *P. parasitica* Emoy2.

Interaction of *AvrRPP4* with RPP4 in *P.p.* Emoy2-infected Col-0 cells leads to rapid and effective resistance responses. In true leaves, RPP4-mediated signalling requires the presence of the defence components *DTH9*, *EDS1*, *PAD4*, *PAL*, *PBS2*, *PBS3*, *SID1* and *SID2*, and salicylic acid accumulation (see text). The relative position and the function of these signalling components in the RPP4 pathway is not known. The *dth9*, *ndr1-1*, *pbs2*, *pbs3*, *rps5-1*, *sid1* and *sid2* mutations also compromise *P. syringae* resistance conferred by one or several CC-NB-LRR genes. PAL is the entry point of the phenylpropanoid pathway leading to the production of salicylic acid and lignin. Inhibition in true leaves of PAL activity by 2-aminoindan-2-phosphonic acid (AIP) compromises RPP4 function (Mauch-Mani and Slusarenko, 1996). In true leaves, salicylic acid plays a role in both the *NPR1*-dependent SAR pathway and the RPP4 pathway, but SAR operates downstream or independently of the RPP4 pathway. In cotyledons (but not in true leaves), RPP4 function is affected in the Col-0 *rps5-1*, *ndr1-1*, and *npr1-1* mutants, indicating that, at least in cotyledons, there may be cross-talk between the TIR-NB-LRR and CC-NB-LRR pathways, and may also suggest that SAR is an integral part of RPP4-mediated resistance in cotyledons but not in true leaves. *EDS1* and *PAD4* share homology to eukaryotic lipases (Falk *et al.*, 1999; Jirage *et al.*, 1999); RPS5 is CC-NB-LRR protein (Warren *et al.*, 1998); *NDR1* is a predicted two-transmembrane (2TM) protein (Century *et al.*, 1997); and *NPR1* is an ankyrin-repeat protein that interacts with a certain class of bZIP transcription factors possibly involved in the activation of *PATHOGENESIS RELATED (PR-1)* genes (Cao *et al.*, 1997; Zhang *et al.*, 1999).



ing that *PAD4* is exclusively part of the *EDS1* pathway (Figure 6).

Low sporulation of *P.p.* Emoy2 and Emwa1 can be observed on cotyledons of wild-type Col-0 plants, and in plants heterozygous at *RPP4*, partial resistance is predominantly expressed in cotyledons (Century *et al.*, 1995; Century *et al.*, 1997; Glazebrook *et al.*, 1997; Holub *et al.*, 1994; data not shown). These observations suggest that *RPP4*-mediated resistance is less effective in cotyledon tissue than in true leaves. On cotyledons of Col-0 plants carrying the *ndr1*, *npr1* and *rps5-1* mutations, a small but statistically significant enhancement of *P. parasitica* sporulation has been observed, while no sporulation or mycelium was detected in true leaves (Table 2). The contrasting phenotypes of the two different tissues suggest a developmental regulation of *RPP4*-mediated resistance. They may also reflect reduced penetrance of *RPP4* resistance in cotyledon tissue, permitting a more sensitive assessment of the involvement of certain defence components. Consequently, the cotyledon-resistance assays may reveal a slight but quantitative involvement of *NDR1*, *NPR1* and the *rps5-1* allele in *RPP4* function (Table 2).

Phenylalanine ammonium lyase (PAL) and cinnamyl alcohol dehydrogenase (CAD) are components of the phenylpropanoid pathway, leading to the synthesis of secondary metabolites such as salicylic acid, lignin, and flavonoids (Mauch-Mani and Slusarenko, 1996). The *in vivo* requirement of PAL and CAD enzymatic activity in Col-0 leaves for full *P. parasitica* Emwa1 resistance functionally implicated salicylic acid and lignin in *RPP4*-mediated resistance (Mauch-Mani and Slusarenko, 1996). In PAL-suppressed Col-0 plants, resistance to *P.p.* Emwa1 was restored by exogenous salicylic acid application, suggesting that salicylic acid production is a major function of PAL in resistance to *P. parasitica* (Mauch-Mani and Slusarenko, 1996). Indeed, removal of salicylic acid by expression of the bacterial salicylate hydroxylase gene *NahG* impairs *RPP4* function in Col-0 true leaves (Nawrath and Métraux, 1999; Table 2). The *sid1* and *sid2* mutants that are impaired in the pathway leading to salicylic acid biosynthesis show impaired *RPP4* function in true leaves (Nawrath and Métraux, 1999). In contrast, the salicylic acid response element *NPR1*, which is required for the induction of systemic acquired resistance (SAR), is not required for *RPP4*-mediated resistance in true leaves (Table 2). Furthermore, foliar application of the salicylic acid analogue benzothiadiazole (BTH; Lawton *et al.*, 1996), induced SAR in wild-type Col-0 plants and in *eds1*, *pad4*, *pbs2* and *pbs3* mutants (but not in *npr1* mutants; Table 2). These results indicate that SAR operates downstream or independently of the *RPP4* pathway, and suggest a dual role for salicylic acid in both an *NPR1*-dependent pathway leading to SAR and the *RPP4* pathway defined by *EDS1*, *PAD4*, *PBS2* and *PBS3* (Figure 6).

In conclusion, molecular isolation of the *RPP4* gene has allowed us to scrutinize the requirements of a TIR-NB-LRR protein for a broad range of signalling components. Our analyses reveal that, while *RPP4* function requires salicylic acid accumulation and signals most strongly through *EDS1*, *PAD4*, *PBS2*, and *PBS3*, other mechanisms regulated by *NPR1* and *NDR1* impinge on *RPP4* function, suggesting a degree of cross-talk in *R* gene-mediated plant defence signalling.

Experimental procedures

Mapping of RPP4

RPP4 for resistance to *P. parasitica* race Emoy2 has been mapped on a 23 cM segment of chromosome 4 in a cross between Col-5 and Niederzenz (Nd-1; Tör *et al.*, 1994). To confirm and refine the position of *RPP4*, we used an F_2 between Col-0 and Wassilewskija (Ws-0). Ws-0 is resistant to *P.p.* Emoy2, but is susceptible to its natural recombinant variants *P.p.* Emwa1 and Emco5 (Table 4; McDowell *et al.*, 1998). *P.p.* race Emwa1 was therefore used to determine the *RPP4* genotypes of Col-0 \times Ws-0 F_2 seedlings and their F_3 progenies. DNA was isolated from 217 F_2 seedlings, and analysed for *P. parasitica* Emwa1 resistance and linkage to various molecular markers (Figure 1a). Thirty-four plants with recombinations between the *ARA* and *AG* co-dominant cleaved amplified polymorphic sequences (CAPS; Koniczny and Ausubel, 1993) were selected and analysed further (Figure 1a). Four recombinations proximal to *RPP4* were detected with the single sequence-length polymorphism (SSLP) *VRN2*, and 10 and two recombinations were found distal to *RPP4* with the CAPS markers *T6-3* with *FCA8-2*, respectively. No recombinations were found with markers *G4539* and *Col-A* (Figure 1a). Details on the CAPS and SSLP markers are available upon request.

Cloning of Col-0 RPP5 homologues and complementation of RPP4 function

A pCD04541 binary cosmid library containing approximately four Col-0 genome equivalents (courtesy of Ian Bancroft, John Innes Centre, Norwich, UK) was screened with a radiolabelled 5 kb *NcoI/EcoRI* genomic *RPP5* fragment (Parker *et al.*, 1997). DNA gel-blot analysis using *HindIII* and the radiolabelled *RPP5* probe confirmed that the cosmid inserts contained *RPP5*-homologous sequences of predicted sizes. By restriction mapping (*EcoRI*, *BamHI*, *BamHI/SalI*), 36 strong hybridizing cosmids were analysed and placed on the \approx 80 kb Col-0 *RPP5* region (GenBank accession Z97342); 10 cosmids were selected that contained the six (near) full-length *RPP5* homologues (*Col-A*, *Col-B*, *Col-D*, *Col-E*, *Col-F* and *Col-G*). The authenticity of the DNA inserts and the precise genomic map locations of the 10 selected cosmids were determined by direct DNA sequencing of the cosmid border fragments.

To test for complementation of *P.p.* Emoy2 and Emwa1 resistance, the 10 binary cosmids were transferred to the CW-84 recipient line by *Agrobacterium tumefaciens* strain GV3101-mediated transformation (Clough and Bent, 1998). The possible occurrence of recombination in *A. tumefaciens* was verified because most cosmid inserts contained multiple *RPP5*-homologous sequences. Cosmid DNA was re-isolated from *A. tumefaciens*, and the *EcoRI* restriction patterns were analysed in parallel with those from cosmid DNA isolated from *E. coli* DH5 α (*rec⁻*; Gibco/BRL, Paisley, UK), but no evidence for recombination-

induced rearrangements was found. The CW-84 progenitor was selected from a Col-*gl* × Ws-0 F_2 population for the absence of *RPP1*, *RPP2* and *RPP4*, and is therefore universally susceptible to the *P.p.* races Cala2, Emoy2, Emwa1 and Noco2 (described by Botella *et al.*, 1998; Tables 1 and 3). At least 12 independent CW-84 T_1 transformants of each of the 10 cosmids were selected for kanamycin resistance (200 mg l⁻¹), self-pollinated, and 30–60 T_2 seedlings were tested in at least two separate experiments for resistance to several *P.p.* races (Table 1).

Arabidopsis thaliana and *Peronospora parasitica*

Arabidopsis growth and *Peronospora parasitica* propagation, and infections of cotyledons and true leaves, including lactophenol–trypan blue staining, were as described previously (Holub *et al.*, 1994; Parker *et al.*, 1996; Parker *et al.*, 1997). 1-week-old (cotyledons) to 2-week-old (true leaves) seedlings were sprayed with *P. parasitica* conidiospores (4×10^4 ml⁻¹) and incubated at 16°C and high humidity. After 5–7 days the seedlings were analysed for asexual sporulation using a 10× magnifying glass, and by staining with lactophenol–trypan blue to microscopically (100×) assess hyphal growth using phase-contrast optics. Following destaining with chloral hydrate, the lactophenol–trypan blue dye is retained in *P. parasitica* cells and in dead or dying plant cells (including vascular xylem). Induction of systemic acquired resistance (SAR) by foliar application of the salicylic acid analogue benzothiadiazole (BTH) was done as described (30 µM BTH in water; Lawton *et al.*, 1996). Plants were infected with *P. parasitica* 2–4 days after BTH treatment. All *P. parasitica* infections were performed in at least two replicates with 30–60 seedlings.

The following Col-0 mutants and transgenic lines were obtained from our colleagues: Col-0 *npr1-1* (X. Dong, Duke University, Durham, USA); Col-0 *ndr1-1* (B. Staskawicz, University of California, Berkeley, CA, USA); Col-0 *pbs2*, Col-0 *pbs3*, Col-0 *rps5-1* and an F_2 between Col-0 *pbs2* and *Ler* (R. Innes, Indiana University, Bloomington, IN, USA); Col-0 *NahG* (J.-P. Mettraux, University of Fribourg, Fribourg, Switzerland); and Col-0 *pad4-1* (J. Glazebrook, University of Maryland, MD, USA). The *Ler pad4-2* and *rpp5-2* mutants were identified in screens for loss of *P. parasitica* Noco2 resistance (Louise N. Frost and J.E.P., unpublished). The Col-0 *pbs2* and the Col-0 *npr1-1* mutations were introduced into an *RPP5* background by crossing to *Ler* and selecting F_2 individuals with flanking markers (data available on request). The *Ler eds1-2* mutation was introduced into an *RPP4* background by crossing to Col-*gl* (described by Aarts *et al.*, 1998).

Nucleic acid manipulations and yeast two-hybrid analysis

Plant and bacterial DNA isolations, PCR, DNA sequencing, cosmid and plasmid manipulations, restriction digests and yeast two-hybrid protocols were as described previously (Botella *et al.*, 1998; Noël *et al.*, 1999; Parker *et al.*, 1997; Van der Biezen *et al.*, 2000). Direct DNA sequencing of the cosmid inserts was done using standard protocols with 4 µg cosmid DNA isolated from *E. coli* DH5α (Gibco/BRL) with Tip20 columns (Qiagen, Chatsworth, CA, USA) and precipitated with polyethylene glycol (PEG mw4000, Sigma, Dorset, UK).

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References

- Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J. and Parker, J.E. (1998) Different requirements for *EDS1* and *NDR1* by disease resistance genes define at least two *R* gene-mediated signaling pathways in *Arabidopsis*. *Proc. Natl Acad. Sci. USA*, **95**, 10306–10311.
- Bevan, M., Bancroft, I., Bent, E. *et al.* (1998) Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. *Nature*, **391**, 485–488.
- Bittner-Eddy, P.D., Crute, I.R., Holub, E.B. and Beynon, J.L. (2000) *RPP13* is a simple locus in *Arabidopsis thaliana* for alleles that specify downy mildew resistance to different avirulence determinants in *Peronospora parasitica*. *Plant J.* **21**, 177–188.
- Botella, M.A., Parker, J.E., Frost, L.N., Bittner-Eddy, P.D., Beynon, J.L., Daniels, M.J., Holub, E.B. and Jones, J.D.G. (1998) Three genes of the *Arabidopsis RPP1* complex resistance locus recognize distinct *Peronospora parasitica* avirulence determinants. *Plant Cell*, **10**, 1847–1860.
- Cao, H., Glazebrook, J., Clarke, J.D., Volko, S. and Dong, X.N. (1997) The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell*, **88**, 57–63.
- Century, K.S., Holub, E.B. and Staskawicz, B.J. (1995) *NDR1*, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and a fungal pathogen. *Proc. Natl Acad. Sci. USA*, **92**, 6597–6601.
- Century, K.S., Shapiro, A.D., Repetti, P.P., Dahlbeck, D., Holub, E. and Staskawicz, B.J. (1997) *NDR1*, a pathogen-induced component required for *Arabidopsis* disease resistance. *Science*, **278**, 1963–1965.
- Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.
- Cooley, M.B., Pathirana, S., Wu, H.-J., Kachroo, P. and Klessig, D.F. (2000) Members of the *Arabidopsis HRT/RPP8* family of resistance genes confer resistance to both viral and oomycete pathogens. *Plant Cell*, **12**, 663–676.
- Delaney, T.P., Uknes, S., Vernooij, B. *et al.* (1994) A central role of salicylic acid in plant disease resistance. *Science*, **266**, 1247–1249.
- Ellis, J.G., Lawrence, G.J., Luck, J.E. and Dodds, P.N. (1999) The identification of regions in alleles of the flax rust resistance gene *L* that determine differences in gene-for-gene specificity. *Plant Cell*, **11**, 495–506.
- Falk, A., Feys, B.J., Frost, L.N., Jones, J.D.G., Daniels, M.J. and Parker, J.E. (1999) *EDS1*, an essential component of *R* gene-mediated disease resistance in *Arabidopsis*, has homology to eukaryotic lipases. *Proc. Natl Acad. Sci. USA*, **96**, 3292–3297.
- Feys, B.J. and Parker, J.E. (2000) Interplay of signaling pathways in plant disease resistance. *Trends Genet.* **16**, 449–455.
- Feys, B.J., Moisan, L.J., Newman, M.A. and Parker, J.E. (2001) Direct interaction between the *Arabidopsis* disease resistance signaling proteins, *EDS1* and *PAD4*. *EMBO J.* **20**, 5400–5411.
- Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. *Science*, **261**, 754–756.
- Glazebrook, J., Zook, M., Mert, F., Kagan, I., Rogers, E.E., Crute,

- I.R., Holub, E.B., Hammerschmidt, R. and Ausubel, F.M. (1997) Phytoalexin-deficient mutants of *Arabidopsis* reveal that *PAD4* encodes a regulatory factor and that four *PAD* genes contribute to downy mildew resistance. *Genetics*, **146**, 381–392.
- Holub, E.B., Beynon, L.J. and Crute, I.R. (1994) Phenotypic and genotypic characterization of interactions between isolates of *Peronospora parasitica* and accessions of *Arabidopsis thaliana*. *Mol. Plant-Microbe Interact.* **7**, 223–239.
- Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P. and Valent, B. (2000) Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *EMBO J.* **19**, 4004–4014.
- Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., Ausubel, F.M. and Glazebrook, J. (1999) *Arabidopsis thaliana* *PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. *Proc. Natl Acad. Sci. USA*, **96**, 13583–13588.
- Jones, D.A. and Jones, J.D.G. (1996) The role of leucine-rich repeat proteins in plant defences. *Adv. Bot. Res.* **24**, 89–167.
- Konieczny, A. and Ausubel, F.M. (1993) A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. *Plant J.* **4**, 403–410.
- Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T. and Ryals, J. (1996) Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *Plant J.* **10**, 71–82.
- Luck, J.E., Lawrence, G.J., Dodds, P.N., Shepherd, K.W. and Ellis, J.G. (2000) Regions outside of the leucine-rich repeats of flax rust resistance proteins play a role in specificity determination. *Plant Cell*, **12**, 1367–1377.
- Mauch-Mani, B. and Slusarenko, A.J. (1996) Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. *Plant Cell*, **8**, 203–221.
- Mayda, E., Mauch-Mani, B. and Vera, P. (2000) *Arabidopsis dth9* mutation identifies a gene involved in regulating disease susceptibility without affecting salicylic acid-dependent responses. *Plant Cell*, **12**, 2119–2128.
- McDowell, J.M., Dhandaydham, M., Long, T.A., Aarts, M.G.M., Goff, S., Holub, E.B. and Dangl, J.L. (1998) Intragenic recombination and diversifying selection contribute to the evolution of downy mildew resistance at the *RPP8* locus of *Arabidopsis*. *Plant Cell*, **10**, 1861–1887.
- McDowell, J.M., Cuzick, A., Can, C., Beynon, J., Dangl, J.L. and Holub, E.B. (2000) Downy mildew (*Peronospora parasitica*) resistance genes in *Arabidopsis* vary in functional requirements for *NDR1*, *EDS1*, *NPR1* and salicylic acid accumulation. *Plant J.* **22**, 523–529.
- Meyers, B.C., Dickerman, A.W., Michelmore, R.W., Sivaramakrishnan, S., Sobral, B.W. and Young, N.D. (1999) Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. *Plant J.* **20**, 317–332.
- Nawrath, C. and Métraux, J.-P. (1999) Salicylic acid induction-deficient mutants of *Arabidopsis* express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell*, **11**, 1393–1404.
- Noël, L., Moores, T.L., Van der Biezen, E.A., Parniske, M., Daniels, M.J., Parker, J.E. and Jones, J.D.G. (1999) Pronounced intra-specific haplotype divergence at the *RPP5* complex disease resistance locus of *Arabidopsis*. *Plant Cell*, **11**, 2099–2111.
- O'Neill, L.A.J. and Greene, C. (1998) Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects, and plants. *J. Leukocyte Biol.* **63**, 650–657.
- Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D. and Daniels, M.J. (1996) Characterization of *eds1*, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different *RPP* genes. *Plant Cell*, **8**, 2033–2046.
- Parker, J.E., Coleman, M.J., Szabò, V. et al. (1997) The *Arabidopsis* downy mildew resistance gene *RPP5* shares similarity to the Toll and interleukin-1 receptors with *N* and *L6*. *Plant Cell*, **9**, 879–894.
- Parker, J.E., Feys, B.J., Van der Biezen, E.A. et al. (2000) Unravelling *R* gene-mediated disease resistance pathways in *Arabidopsis*. *Mol. Plant Pathol.* **1**, 17–34.
- Parniske, M., Hammond-Kosack, K.E., Golstein, C., Thomas, C.M., Jones, D.A., Harrison, K., Wulff, B.B. and Jones, J.D. (1997) Novel disease resistance specificities result from sequence exchange between tandemly repeated genes at the *Cf-4/9* locus of tomato. *Cell*, **91**, 821–832.
- Thomas, C.M., Jones, D.A., Parniske, M., Harrison, K., Balint-Kurti, P.J., Hatzixanthis, K. and Jones, J.D. (1997) Characterization of the tomato *Cf-4* gene for resistance to *Cladosporium fulvum* identifies sequences that determine recognitional specificity in *Cf-4* and *Cf-9*. *Plant Cell*, **9**, 2209–2224.
- Tör, M., Holub, E.B., Brose, E., Musker, R., Gunn, N., Can, C., Crute, I.R. and Beynon, J.L. (1994) Map positions of three loci in *Arabidopsis thaliana* associated with isolate-specific recognition of *Peronospora parasitica* (downy mildew). *Mol. Plant-Microbe Interact.* **7**, 214–222.
- Van der Biezen, E.A. and Jones, J.D.G. (1998a) The NB-ARC domain: a novel signalling motif shared by plant resistance gene products and regulators of cell death in animals. *Curr. Biol.* **8**, R226–R227.
- Van der Biezen, E.A. and Jones, J.D.G. (1998b) Plant disease resistance proteins and the gene-for-gene concept. *Trends Biochem. Sci.* **23**, 454–456.
- Van der Biezen, E.A., Sun, J., Coleman, M.J., Bibb, M.J. and Jones, J.D.G. (2000) *Arabidopsis RelA/SpoT* homologs implicate (p)ppGpp in plant signaling. *Proc. Natl Acad. Sci. USA*, **97**, 3747–3752.
- Van der Vossen, E.A., Van der Voort, J.N., Kanyuka, K., Bendahmane, A., Sandbrink, H., Baulcombe, D.C., Bakker, J., Stiekema, W.J. and Klein-Lankhorst, R.M. (2000) Homologues of a single resistance-gene cluster in potato confer resistance to distinct pathogens: a virus and a nematode. *Plant J.* **5**, 567–576.
- Warren, R.F., Henk, A., Mowery, P., Holub, E. and Innes, R.W. (1998) A mutation within the leucine-rich repeat domain of the *Arabidopsis* disease resistance gene *RPS5* partially suppresses multiple bacterial and downy mildew resistance genes. *Plant Cell*, **10**, 1439–1452.
- Warren, R.F., Merritt, P.M., Holub, E. and Innes, R.W. (1999) Identification of three putative signal transduction genes involved in *R* gene-specified disease resistance in *Arabidopsis*. *Genetics*, **152**, 401–412.
- Zhang, Y.L., Fan, W.H., Kinkema, M., Li, X. and Dong, X.N. (1999) Interaction of *NPR1* with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the *PR-1* gene. *Proc. Natl Acad. Sci. USA*, **96**, 6523–6528.
- Zhou, N., Tootle, T.L., Tsui, F., Klessig, D.F. and Glazebrook, J. (1998) *PAD4* functions upstream from salicylic acid to control defense responses in *Arabidopsis*. *Plant Cell*, **10**, 1021–1030.

The GenBank accession number of *RPP4* is AF440696