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Two TIR:NB:LRR genes are required to specify resistance to *Peronospora parasitica* isolate Cala2 in *Arabidopsis*

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Summary

Resistance responses that plants deploy in defence against pathogens are often triggered following a recognition event mediated by resistance (*R*) genes. The encoded R proteins usually contain a nucleotide-binding site (NB) and a leucine-rich repeat (LRR) domain. They are further classified into those that contain an N-terminal coiled coil (CC) motif or a Toll interleukin receptor (TIR) domain. Such *R* genes, when transferred into a susceptible plant of the same or closely related species, usually impart full resistance capability. We have used map-based cloning and mutation analysis to study the recognition of *Peronospora parasitica* (*RPP*)*2* (*At*) locus in *Arabidopsis* accession Columbia (Col-0), which is a determinant of specific recognition of *P. parasitica* (*At*) isolate Cala2. Genetic mapping located *RPP2* to a 200-kb interval on chromosome 4, which contained four adjacent TIR:NB:LRR genes. Mutational analysis revealed three classes of genes involved in specifying resistance to Cala2. One class, which resulted in pleiotropic effects on resistance to other *P. parasitica* (*At*) isolates, was unlinked to the *RPP2* locus; this class included *AtSGT1b*. The other two classes were mapped within the interval and were specific to Cala2 resistance. Representatives of each of these classes were sequenced, and mutations were found in one or the other of two (*RPP2A* and *RPP2B*) of the four TIR:NB:LRR genes. *RPP2A* and *RPP2B* complemented their specific mutations, but failed to impart resistance when present alone, and it is concluded that both genes are essential determinants for isolate-specific recognition of Cala2. RPP2A has an unusual structure with a short LRR domain at the C-terminus, preceded by two potential but incomplete TIR:NB domains. In addition, the RPP2A LRR domain lacks conserved motifs found in all but three other TIR:NB:LRR class proteins. In contrast, RPP2B has a complete TIR:NB:LRR structure. It is concluded that RPP2A and RPP2B cooperate to specify Cala2 resistance by providing recognition or signalling functions lacked by either partner protein.

Keywords: *Arabidopsis*, digenic resistance, *R* genes, downy mildew, NB:LRR, *Peronospora*.

Introduction

Genetic variation between plants of different genotype, following challenge by biotrophic pathogens, is often expressed as either compatibility or incompatibility. The former circumstance is characterised by pathogen development and reproduction, with little visible cellular response by the host. In the latter circumstance, the pathogen is recognised by the plant, and processes are triggered that impede its further development. This incompatibility is often associated with restricted rapid host cell death. Variation in host–parasite interactions can be attributed to the presence or absence of specific resistance (*R*) genes, whose products enable plants to respond to pathogens delivering matching, specific avirulence (*avr*) gene products (Flor, 1971). The so-called 'gene-for-gene' hypothesis predicts that compatibility and, therefore, disease occurs if either of the matching gene pair products is absent from the interaction.

R genes have been cloned from a range of plants, and a consistency of structure has been revealed (Ellis *et al.*, 2000). The majority of these genes encode proteins that contain a nucleotide-binding site (NB) domain, which occurs in diverse proteins with ATP/GTP-binding activity (Bent, 1996), and a leucine-rich repeat (LRR) domain predicted to mediate protein–protein interactions (Kobe and Deisenhofer, 1994). The NB domain is often associated with conserved ARC motifs, which show similarity to the nematode CED-4 and the mammalian APAF-1 proteins that regulate cell death (Van der Biezen and Jones, 1998). *R* genes can be further divided into two subclasses: one includes genes whose proteins contain a coiled coil (CC) motif at their N-terminus (e.g. recognition of *Peronospora parasitica* (*RPP*)*8*, McDowell *et al.*, 1998; *RPP13*, Bittner-Eddy *et al.*, 2000), the other includes genes whose proteins contain an N-terminal domain (Toll interleukin receptor (TIR) domain), with similarity to the Toll domain in *Drosophila* and the IL-1R domain in mammals (*RPP5*, Parker *et al.*, 1997; *RPP1*, Botella *et al.*, 1998). The products of these genes are predicted to be located in the cytoplasm. Other *R* genes with extracellularly located LRR domains are membrane-anchored, and either lack an obvious protein domain involved in signalling (e.g. *Cf9*, Jones *et al.*, 1994) or contain a cytoplasmically located serine–threonine kinase domain (e.g. *Xa21*, Wang *et al.*, 1998). The gene *Pto* from tomato represents an exception. This a cytoplasmic serine–threonine kinase (Martin *et al.*, 1993) which, however, requires the linked gene *Prf*, an NB:LRR gene, to enable an incompatible response to isolates of *Pseudomonas syringae* delivering the *AvrPto avr* gene product (Salmeron *et al.*, 1996).

In some cases, it has been demonstrated that a direct physical effect of the Avr protein on the R protein enables the incompatible response. This is the case for the *Rx* gene from potato that confers resistance to potato virus X (PVX). The viral coat protein causes the relaxation of intramolecular interactions within Rx, which, in turn, triggers resistance (Moffet *et al.*, 2002). R proteins may also be components of complexes, for example, RPM1 and RPS2 from *Arabidopsis* are associated with the RIN4 protein. AvrRpt2 cleaves RIN4 and triggers resistance through the release of RPS2 from the complex (Axtell and Staskawicz, 2003; Mackey *et al.*, 2003), whereas the presence of RIN4 is required for RPM1 resistance, perhaps through an interaction between AvrRpm1 and RIN4, resulting in the release of RPM1 (Mackey *et al.*, 2002).

Holub *et al.* (1994) examined the inheritance of resistance to *Peronospora parasitica* (*At*) isolate Cala2 in *Arabidopsis* accession Columbia (Col-0). From a thorough characterisation of the interaction phenotype, including host cell response and quantity of pathogen sporulation, Holub *et al.* (1994) concluded that the expression of resistance to Cala2 in Col-0 was allele dosage-dependent, being under the control of a partially dominant *R* allele (*RPP2*) at a single locus. Using 100 F_3 families from a Col-0 × Neiderzens (Nd-1) cross, Tör *et al.* (1994) mapped *RPP2* to a single locus on chromosome 4, 4.8 cM below the morphological marker *Agamous* and 9.1 cM above the RFLP locus *B9* (Figure 1a).

Here, we report that resistance to *P. parasitica* (*At*) isolate Cala2, specified by the *RPP2* locus in Col-0, requires the

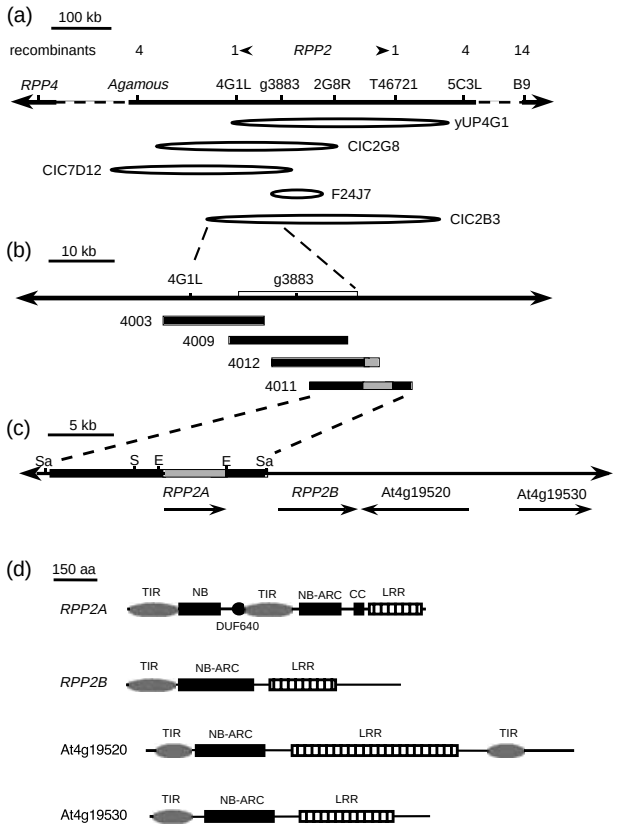

Figure 1. Genetic map of the *RPP2* locus and protein analysis.
(a) Molecular markers used to map *RPP2*. The number of recombinants identified in the F_3 and F_9 RIL mapping populations is given above the respective loci. Dashed lines indicate regions not drawn to scale. The arrowed bar shows the maximum interval encompassing *RPP2*. The YAC clone contig map was generated by Schmidt *et al.* (1996). The F24J7 BAC clone was sequenced by the International *Arabidopsis* sequencing project (http://www.mips.biochem.mpg.de/).
(b) Chromosome walk using lambda clones. A walk was initiated using 4G1L and g3883 as hybridisation probes to a Col-0 DNA lambda library. Lambda clone 4011 contains the complete *RPP2A* gene as represented by the grey box.
(c) Detail from the fragment of F24J7 containing *RPP2A* and the three linked resistance-like genes. The relative position of lambda clone 4011 and sites for *Sal*I (Sa), *Spe*I (S) and *Eco*RI (E) are shown. The 8.8-kb *Spe*I–*Sal*I fragment containing *RPP2A* was used in transformation experiments. The two *Eco*RI sites delineate the 4.7-kb DNA fragment used to diagnose the presence or absence of the *RPP2A* transgene in T_2 progeny of *FN2* mutant (Figure 2). The arrows indicate the orientation of each gene.
(d) Representation of protein domains based on putative functional motifs. The Conserved Domain Architecture Retrieval Tool (CDART; http://www.ncbi.nlm.nih.gov/BLAST/) and visual inspection of the amino acid sequences were used to determine the relative positions of putative functional motifs (see text and Figures 3 and 4 for more details). Undecorated lines depict regions with unknown function.

presence of two resistance determinants, both of which reside at the *RPP2* locus and are members of the TIR:NB:LRR class of *R* gene.

Results

Mutational analysis reveals that resistance to Cala2 requires the presence of two different R *alleles at the* RPP2 *locus*

Two populations of mutagenised Col-0 plants were screened to detect variation for interaction phenotype following inoculation with Cala2: one population had been treated with fast neutrons (FNs) and the other with ethylmethane sulfonate (EMS; see Experimental procedures). M_2 seedlings were scored 7 days after inoculation for presence or absence of asexual sporulation. Plants supporting sporulation were grown to maturity, and seeds were collected. M_3 plants from the selected mutants were re-tested with Cala2 to confirm that the plants originally selected represent true phenotypic variants. Seven EMS and three FN Cala2-susceptible mutants were obtained.

To determine which of the mutations were specific to Cala2 recognition, each mutant line was inoculated with a panel of *P. parasitica* (*At*) isolates. Resistance to each of these isolates in Col-0 is conferred by *R* genes, which are distinct from *RPP2*. Hence, an alteration in the interaction phenotype observed, following inoculation with these isolates, would imply that the mutation was in a gene involved in the disease-resistance response, common to both *RPP2*, and one or more other *R* genes. Majority of the mutants identified, following inoculation with *RPP2*, exhibited a wild-type resistance phenotype to all the other isolates tested, while the remaining were susceptible to Cala2 (data not shown). Two pleiotropic mutants (*FN1* and *EMS1*) were identified. Mutant *FN1* showed a susceptible reaction following inoculation with all isolates tested, and it was concluded that this was probably a mutant of a gene involved in the signalling pathway required for the function of several different *R* genes. Subsequently, Tör *et al.* (2002) named the mutation in line FN1 enhanced downy mildew susceptibility (*edm*) *1* and showed the gene (*AtSGT1b*) to be an orthologue of yeast SGT1. Mutant *EMS1* exhibited a less than fully compatible phenotype following inoculation with isolates Cala2, Emwa1 and Cand5, characterised by a moderate level of sporulation.

All mutants were back-crossed to Col-0 to determine the number of genes segregating for the observed phenotypes following inoculation with Cala2. The recovery of the wild-type resistant phenotype in the F_1 progeny was consistent with the recessive nature of the mutations, and it confirmed that the back-crosses were successful (Table 1). The small number of F_1 seedlings, exhibiting a low level of sporulation in the crosses between Col-0 and some of the mutants (*EMS1*, *EMS5*, *EMS6*, *EMS7*, *FN1*, *FN2* and *FN3*) could be

Table 1 Segregation of interaction phenotypes among F_1 and F_2 progeny from mutants crossed with Col-0, Nd-1 and the *FN2* mutant following inoculation with *Cala2*

	× Col-0 (N:L:M:H)[a]			× Nd-1 (N:L:M:H)		× FN2 (N:L:M:H)	
	F_1	F_2	χ^{2b} (3 : 1)[c]	F_1	F_2	F_1	F_2
Class 1							
FN1 (H)	133 : 2 : 1 : 0	208 : 6 : 2 : 45	3.1	7 : 31 : 6 : 1	15 : 20 : 0 : 19	13 : 27 : 0 : 0	137 : 16 : 0 : 32
EMS1 (L5)	80 : 1 : 0 : 0	45 : 4 : 1 : 4	2.0	4 : 18 : 2 : 0	11 : 25 : 0 : 13	4 : 1 : 0 : 0	32 : 4 : 0 : 14
Class 2A							
EMS2 (M)	7 : 0 : 0 : 0	76 : 6 : 2 : 7	3.5	1 : 9 : 8 : 4[d]	1 : 0 : 0 : 43[d]	0 : 0 : 0 : 36	1 : 2 : 20 : 41[d]
EMS3 (LM)	91 : 0 : 0 : 0	102 : 8 : 2 : 14	2.4	0 : 0 : 1 : 7	8 : 6 : 0 : 63[d]	0 : 0 : 2 : 38	1 : 8 : 11 : 38[d]
FN2 (M)	43 : 1 : 0 : 0	118 : 8 : 0 : 32	0.008	0 : 5 : 4 : 30	1 : 5 : 0 : 40[d]	–	–
FN3 (M)	42 : 1 : 0 : 0	55 : 5 : 0 : 8	1.3	1 : 0 : 3 : 28[d]	0 : 14 : 1 : 42	2 : 5 : 1 : 23[d]	15 : 1 : 0 : 121[d]
Class 2B							
EMS4 (M)	36 : 0 : 0 : 0	70 : 16 : 3 : 26	12.2	0 : 3 : 1 : 2	0 : 0 : 0 : 70	2 : 16 : 1 : 0	Not tested
EMS5 (M)	47 : 8 : 0 : 0	112 : 16 : 1 : 13	1.1	0 : 0 : 6 : 9	4 : 4 : 0 : 33[d]	10 : 21 : 0 : 0	87 : 18 : 0 : 102
EMS6 (H)	42 : 2 : 0 : 0	69 : 13 : 5 : 14	2.4	0 : 1 : 2 : 11	2 : 3 : 0 : 55[d]	5 : 14 : 0 : 0	56 : 20 : 0 : 99
EMS7 (M)	17 : 2 : 0 : 0	65 : 5 : 0 : 15	0.1	7 : 1 : 1 : 7[d]	0 : 13 : 0 : 70	6 : 15 : 0 : 0	54 : 8 : 0 : 103

[a]Interaction phenotype according to Holub *et al.* (1994). The asexual sporulation of the isolate is classified according to the average number of sporangiophores per cotyledon as: light (L) = 1–10, medium (M) = 12–17 and high (H) = >17. The abbreviation N indicates no asexual sporulation.

[b]χ^2 for 1 degree of freedom $P < 0.05$ is 3.84.

[c]For the calculation of χ^2. Resistant class, plants displaying the N interaction phenotype; susceptible class, plants displaying the L, M and H interaction phenotypes.

[d]Low numbers of offspring showing no asexual sporulation are because of escape from infection.

attributed to the fact that a low proportion of seedlings of Col-0 may occasionally exhibit this phenotype following inoculation with Cala2. With the exception of EMS4, segregation of Cala2 resistance (3 : 1 resistant:susceptible) among F_2 seedlings was consistent with mutations in single alleles at one locus (Table 1). In contrast, the F_2 segregation data from the cross with EMS4 were characterised by the occurrence of a high proportion of seedlings exhibiting a low intensity of sporulation (L), and ratios between seedlings placed in resistant and susceptible categories were significantly different from expectation, if the mutation was in a single allele at one locus.

Each mutant was also crossed with Nd-1 to determine if the mutations affecting resistance to Cala2 were at the *RPP2* locus. Nd-1 is susceptible to Cala2 and therefore lacks alleles effective in the recognition of Cala2 at the *RPP2* locus. Complementation of the Cala2 recognition phenotype in F_1 progeny would indicate that the mutation did not lie within the *RPP2* locus, but rather in another locus required for its function. Segregation data for reponses to inoculation with Cala2 are presented in Table 1. Two classes of mutants were distinguishable: Class 1 contains the mutants *FN1* and *EMS1*, which, when crossed with Nd-1, resulted in F_1 progeny that were more resistant to Cala2 than the parents, consistent with these mutations not taking place in *RPP2*; Class 2 contains the mutants *EMS2*, *EMS3*, *EMS4*, *EMS5*, *EMS6*, *EMS7*, *FN2* and *FN3*, which, when crossed with Nd-1, resulted in fully susceptible F_1 individuals. Lack of complementation indicated that these lines were likely to contain a mutation allelic to the susceptibility determinant in Nd-1, confirmed by the lack of segregation for resistance among F_2 progeny (Table 1). Hence, Class 2 mutant lines contained mutations at the *RPP2* locus.

To determine if the Class 2 mutants were allelic, they were all crossed with the Class 2 *FN2* mutant. Complementation of the Cala2 recognition phenotype in the F_1 progeny of a mutant $\times$ FN2 cross would indicate that the mutations were not allelic. Conversely, lack of complementation would demonstrate that the mutations were in the same allele. Intriguingly, only the F_1 progeny resulting from FN2, crossed to EMS2, EMS3 and FN3, were susceptible and showed no segregation for resistance among F_2 progeny (Table 1). This demonstrated that the mutations in this group (Class 2A) are allelic. F_1 progeny from the crosses with the other Class 2 (Class 2B) mutants exhibited a higher level of resistance than either parent, which indicated that these lines carry a mutation allelic to a component of resistance missing from Nd-1, but not allelic to *FN2*. As expected, the F_1 data for crosses of Class 1 mutants to FN2 resulted in resistant offspring, confirming that the mutations were in alleles at different loci (Table 1).

Hence, the mutational analyses revealed that at least two linked genes at the *RPP2* locus in Col-0 are required for isolate-specific recognition of Cala2. The susceptible accession Nd-1 lacks alleles capable of recognising Cala2 for either of these genes at the *RPP2* locus.

Fine-scale mapping of the RPP2 *locus in Columbia*

A set of 200 F_9 recombinant inbred (RI) lines from the Col-0 $\times$ Nd-1 cross was used to develop a range of new dimorphic RFLP and co-dominant amplified polymorphic sequence (CAPS) markers in the mapping interval defined by Tör *et al*. (1994). These enabled *RPP2* to be located within an interval defined between the CAPS marker 4G1L and the RFLP marker T46721. *RPP2* co-segregated with the CAPS marker g3883 and RFLP marker 2G8R (Figure 1a). Markers 4G1L and 2G8R were derived from YACs YUP4G1 and ClC2G8, respectively. This anchored the mapping interval to the physical contig of chromosome 4 created by Schmidt *et al*. (1996). A walk was initiated from the flanking markers with lambda clones. It was revealed that λ4011 (Figure 1b) contained an '*R*-gene-like' sequence, which encoded a protein with 64% identity to the RPP5 protein (Parker *et al*., 1997) within the TIR:NB domain. This gene was therefore a candidate for one of the components of Cala2 resistance.

A Southern blot, containing genomic DNA from all the selected mutants restricted with *Eco*R1, was probed with the candidate gene cloned from the *RPP2* mapping interval (data not shown). This experiment revealed that there was a shift in the expected 4.7-kb *Eco*R1 fragment (Figure 1c) in the track containing DNA from the Class 2A *FN2* mutant (Figure 2). Hybridisation with PCR probes, representing smaller parts of the candidate gene, suggested that the deletion or re-arrangement did not extend beyond the end of the gene (data not shown). The candidate gene from the Class 2A EMS mutant *EMS2*, that had previously been shown to be allelic to *FN2*, was

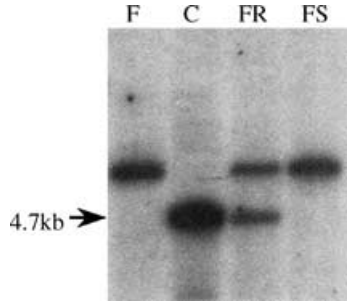

Figure 2. *RPP2A* complements the mutation in FN2 to confer Cala2 resistance.
Southern blot analysis using a *RPP2A*-specific probe was performed on *Eco*RI-digested DNA from wild-type Col-0 (lane C), FN mutant line FN2 (lane F) and two T_2 progeny (lane FR and FS), resulting from transformation of FN2 with an 8.8-kb *Spe*I–*Sal*I fragment containing *RPP2A*. T_2 progeny FR is resistant to Cala2 because of the presence of *RPP2A* (arrowed band), whilst T_2 progeny FS is susceptible to Cala2 in the absence of *RPP2A*.

sequenced. This revealed a single C-to-T substitution in position +457, resulting in an in-frame stop codon, and thereby a truncated protein.

Identification of RPP2A

An 8.8-kb *Spe*l–*Sal*I fragment from λ4011 (Figure 1c), which carried only the candidate gene, was subcloned into a cosmid binary vector to generate c4118. This clone was used to transform two Cala2-susceptible RI lines from the Col-0 × Nd-1 cross and the Class 2A mutant *FN2*. T_2 progeny from five independent FN2 transgenic lines (30 plants per line on average) were found to segregate 3 : 1 (resistant:susceptible) for response to Cala2. There was an absolute correlation between Cala2 resistance and BASTA tolerance (the marker used to select transformants). Figure 2 shows a Southern blot containing DNA from Cala2-resistant (FR) and -susceptible (FS) T_2 progeny from an FN2 transgenic line. The 4.7-kb *Eco*RI fragment within the 8.8-kb *Spe*l–*Sal*I fragment was only detected in Cala2-resistant transgenic plants. However, the equivalent RI line transformants produced only Cala2-susceptible progeny as did FN2 plants transformed with the binary vector alone. These data are consistent with the segregation data from crosses with the mutant lines, which indicated that Nd-1 lacks alleles capable of recognising Cala2 at both the two genes within the *RPP2* locus. These results demonstrated that the gene cloned from the *RPP2* mapping interval has a functional role in Cala2 recognition, but is only one of at least two components. This gene was called *RPP2A*.

Identification of the second component of Cala2 resistance at the RPP2 *locus*

The mapping interval containing the *RPP2* locus spanned approximately 200 kb (Figure 1a). To identify the second component required for Cala2 resistance, we selected F_2 progeny from a Col-0 × Nd-1 cross, which were susceptible to Cala2. These F_2 progeny would lack effective alleles of either or both putative genes at the *RPP2* locus. We screened 180 of these F_2 progeny for those that contained *RPP2A* from Col-0, reasoning that any such offspring must contain a recombination event between *RPP2A* and a second component. No such individuals were obtained. Therefore, given the size of the population available, the additional gene or genes required for Cala2 resistance lay too close to *RPP2A* to be separated by a recombination event.

Concurrently, analysis of the genes within the mapping interval revealed three other NB:LRR genes (*At4g19510*, *At4g19520* and *At4g19530*; Figure 1c,d), which formed a cluster with *RPP2A* (http://www.arabidopsis.org). As we were unable to separate *RPP2A* from the additional component required for Cala2 resistance, these genes were all likely candidates. Therefore, we sequenced these genes from the four Class 2B mutants. Mutations were only found in *At4g19510*, the gene immediately adjacent to *RPP2A*. These mutations all resulted in amino acid substitutions (see below).

At4g19510 was subcloned on a *Hpa*I fragment from bacterial artificial chromosome (BAC) clone F24J7 into the binary vector, which was then used to transform mutant *EMS5*. T_2 offspring from two independent transformants revealed segregation for response to Cala2 (3 : 1 resistant: susceptible); BASTA tolerance co-segregated with Cala2 resistance. Therefore, AT4g19510 is the second component required for Cala2 resistance at the *RPP2* locus, and was called *RPP2B.*

The protein encoded by RPP2A

The 8.8-kb *Spe*l–*Sal*I genomic c4118 insert containing *RPP2A* was sequenced. Annotation of this gene suggested that it contained four introns (http://www.arabidopsis.org). This was confirmed by sequencing *RPP2A* cDNA (see Experimental procedures). *RPP2A* is predicted to encode a cytoplasmic protein consisting of 1308 amino acids (Figure 3).

Comparison of RPP2A with proteins in the GenBank database revealed that it is most similar to RPP5 (Parker *et al.*, 1997). The sequence similarities between RPP2A and RPP5 in the TIR and NB domains are shown in Figure 3. RPP2A shares 69% identity with RPP5 in the TIR domain, while more than 80% identity is evident between the two proteins in the conserved motifs appearing in the NB domain (the pre-P-loop, P-loop, kinase 2 and kinase 3a). The overall identity between them in the TIR:NB region (residues 1–320 for *RPP2A* and 1–326 for *RPP5*) is 64%. The NB domain of RPP2A, unlike that of RPP5, does not contain obvious ARC motifs, which are associated with most NB domains in R proteins (Meyers *et al.*, 1999; Van der Biezen and Jones, 1998). RPP2A is also unique amongst disease-resistance proteins in that it contains a DUF640 motif. The function of this motif is unknown, but it has been found in a small number of other plant proteins in the PFAM database. Surprisingly, C-terminal to the DUF640 motif, a second TIR domain is found in RPP2A (Figure 3). Furthermore, this TIR domain is followed by an additional NB domain, which is associated with ARC motifs. However, this additional NB domain lacks a recognisable pre-P-loop and P-loop motif. Overall, this second TIR:NB region only shows 25% identity to RPP5.

Two additional surprising elements were revealed in the last third of the RPP2A protein. First, although the alignment with RPP5 seems to classify *RPP2A* within the TIR:NB:LRR subclass of disease-resistance genes, the predicted protein also contains a putative CC domain comprising four heptad motifs (Figure 3). Second, the LRR domain

is located at the end of the protein and consists of only seven repeats.

The protein encoded by RPP2B

The *RPP2B* gene (At4g19510) is 5297 bp and lies adjacent to *RPP2A* on chromosome 4. Two different gene models, with fundamentally different C-termini, exist for At4g19510 as a result of different intron/exon predictions (http://www.arabidopsis.org; http://www.mips.biochem.mpg.de/). To determine which, if any, gene model is correct, full-length cDNA was obtained and its sequence was determined (see Experimental procedures). The cDNA sequence revealed that neither model was correct. For example, putative introns 4 and 6 (http://www.arabidopsis.org) were retained in all *RPP2B* cDNAs sequenced, thereby resulting in a predicted TIR:NB:LRR protein of 1207 amino acids (Figure 4). The structure of RPP2B differs markedly from RPP2A. There is no duplication of the TIR:NB region. In mutant *EMS7*, a glycine-to-glutamic acid change occurred in the pre-P-loop (Figure 4). The LRR domain contains 14 clearly defined repeats, of 21–24 amino acids, that conform to previous consensus sequences. Mutant *EMS6* contains a phenylalanine residue in place of a conserved leucine within the β-turn β-loop of the sixth LRR (Figure 4). The LRR domain is separated from the C-terminus by 282 amino acids, which contains no obvious functional motifs. However, the amino acid changes in mutants *EMS4* (glutamic acid to lysine) and *EMS5* (threonine to methionine) occur within this region (Figure 4), immediately adjacent to the last of the 14 defined LRRs. BLASTP analyses, using the whole C-terminal domain, revealed that these mutations lay in a region conserved amongst a subset of *Arabidopsis* TIR:NB:LRR proteins. This region has been annotated as either motif 8 or motif 25 by Meyers *et al.* (2003).

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TIR-1 DOMAIN
1   MAASF-CGSRRYDVFPSFSKVDVRRSFLAHLLKELDRRLINTFT
1   ::::SSS:R:::::::::G:::KT::S:::A::GKS:::I
    DHGMERNLPIDAELLSAIAESRISIVIFSKNYASSTWCLDELVE
    :::I::SRT:AP::I:::R:A:::::::::::::::::N::::
    IHTCYKELAQIVVPVFFNVHPSQVKKQTGEFGKVFGKTC---KG
    ::K:FND:G:M:I:::YD:D::E:R:::::::::E:::EVS:D
    K-PENRKLRWMQALAAVAN
    :Q:GDQ:Q::V:::TDI::

147 IAGYDLQNWPDEAVMIEMVADDVSKKLFKSSNDFS
151 :::E::L:G:N::H:V:KISN:::N::ITR:KC:D

NB DOMAIN
182 DIVGIEAHLEAMSSILRLKSEKARMVGISGPSGIGKTTIAKALF
187 :F::::::I::IK:V:C:E:KE::::::W:Q:::::S::GR:::
    SKLSPQFHLRAFVTYKRTNQDDY-DMKLCWIEKFLSEILGQKDL
    :Q::S:::H:::L:::S:SGS:VSG:::S:QKEL:::::::::I
    KVLDLGAVEQSLMHKKVLIILDDVDDLELLKTLVGQTGWFGFGS
    :IEHF:V:::R:N::::::L:::::N::F::::::KAE:::S::
    RIVVITQD
    ::I:::::

321 RQLLKAHDINLIYEVAFPSAHLALEIFCQSAFGKIYPPSDFREL
    SVEFAYLAGNLPLDLRVLGLAMKGKHREEWIEMLPRLRNDLDGK
    FKKTLRNYLPVIRKRVSNEEGGREKLKKGNKKLDLDEEFPGGEI
    YSDEIPSPTSNWKDTDDFDSGDIIPIIADKSTTIIPNRRHSND

DUF640
495 DWCSFCEFLRNRIPPLNPFKCSANDVIDFLRTRQVLGSTEALVD
    RLIFSSEAFGIKPEENPFRSQAVTSYLKAARDM

TIR-2 DOMAIN
572 TREKECILVFSCHDNLDVDETSFIEAISKELHKQGFIPLTYNLL
    GRENLDEEMLYGSRVGIMILSSSYVSSRQSLDHLVAVMEHWKTT
    DLVIIPIYFKVRLSDICGLKGRFEAAFLQLHMSLQEDRVQKWKA
    AMSEIVS

711 IGGHEWTKGSQFILAEEVVRNASLRLYLKSSKNLLGILALLNHS
    QSTDVEIMGIWGIAGIVAFPSAHLALEIFCQSAFGKIYPPSDFR
    ELSVEFAYLAGNLPLDLRVDFHLMCQMKRPRQLREDFISKLFGE
    EKGLGASDVKPSFMRDWFHK

NB-ARC DOMAIN
840 KTILLVLDDVSNARDAEAVIGGFGWFSHGHRIILTSRSKQVLVQ
    CKVKKPYEIQKLSDFESFRLCKQYLDGENPVISELISCSSGIPL
    ALKLLVSSVSKQYITNMKDHLQSLRKDPPTQIQEAFRRSFDGLD
    ENEKNIFLDLACFF

986 RGQSKDYAVLLLDACGFFTYMGICELIDESLISLVDNKIEMPIP
    FQDMGRIIVHEEDEDPCERSRLWDSKDIVDVLTNNSGTEAIEGI
    FLDASDLTCELSPTVFGKMYNLRLLKFYCSTSGNQCKLT

CC MOTIF
1113 LPHGLDT   LPDELSL   LHWENYP   LVYLPQK

LRR DOMAIN
1141 FNP   VNLV | E  LNM  PY | SNME  KLW  EG
     KKN   LEKL | KN  IKL  SH | SREL  TDI  LM
     LSE   ALNL | EH  IDL  EG | CTSL  IDV  SMS
     IPC   CGKL | VS  LNM  KD | CSRL  RSL  PS
     MVD   LTTL | KL  LNL  SG | CSEF  EDI  QD
     FAP     NL | EE  IYL  AG | T SI  REL  PLS
     IRN   LTEL | VT  LDL  EN | CERL  QEM  PSL
cons aXX   aXXL | XX  LXL  XX | CXXL  XXa  XXX

1301 PVEIIRRT
```

Figure 3. Predicted structural domains and deduced amino acid sequence of RPP2A.

Structural domains were predicted from RPP2A sequence using CDART (http://www.ncbi.nlm.nih.gov/BLAST/) and visual inspection. A comparison between RPP2A and RPP5 (Parker *et al.*, 1997) is shown for the first TIR:NB region of RPP2A, where identity between the two proteins is 64%. The RPP5 sequence is aligned below RPP2A. A colon indicates identical residues, while hyphens indicate gaps introduced to maximise the sequence alignment. CDART analysis predicted two TIR domains, TIR-1 (1–146) and TIR-2 (572–710); two NB domains, one without (182–320) and one with associated ARC motifs (840–985); a DUF640 motif (495–571) and a LRR domain between amino acids 1141–1300. A putative CC motif between 1113 and 1140 was revealed by visual inspection. Conserved motifs found within TIR and NB-ARC domains (Meyers *et al.*, 1999; Van der Biezen and Jones, 1998) are overscored. The seven repeats comprising the LRR domain are aligned over a consensus (cons) sequence. Amino acids were included in the consensus if they comprised 50% or more of the residues at a given position. 'X' indicates any amino acid and 'a' indicates any of the hydrophobic amino acids A, F, I, L, M or V. Vertical lines frame the putative beta-strand/beta-turn subdomain (Jones and Jones, 1997). The boxed sequence DLEGCTSL within the third LRR is conserved between RPP2A, RPP2B and the protein encoded by *At4g19530*.

Conserved regions within the LRR domains of RPP2A *and* RPP2B

As proteins encoded by *RPP2A* and *RPP2B* cooperate to specify Cala2 resistance, we analysed their structure for common protein motifs. We could not detect significant

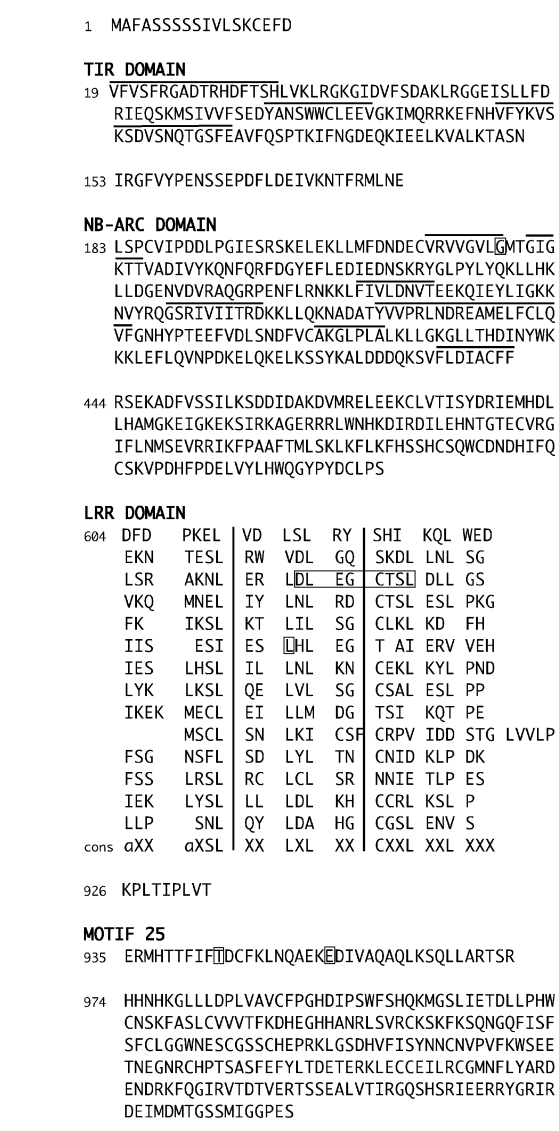

Figure 4. Predicted structural domains and deduced amino acid sequence of RPP2B.
Structural domains were predicted from RPP2B sequence and conserved motifs highlighted as for RPP2A (see Figure 3 for details). CDART analysis predicted single TIR (19–152) and NB-ARC (183–443) domains, and an LRR domain consisting of 14 repeats between amino acids 641–925. Boxed single amino acids show those altered in the four Class 2B mutants sequenced.

homology between *RPP2A* and *RPP2B* outside conserved motifs (e.g. P-loop) within functional domains, suggesting that these genes did not evolve from one another by a simple duplication event. However, two conserved features were apparent within the LRR domains. The amino acid immediately following the β-strand/β-turn motif is conserved between the two proteins and is S, S, C, C, C, T, C in the first seven LRRs in each case (Figures 3 and 4). Furthermore, within the third LRR, the sequence DLEGCTSL is completely conserved. However, no other significant homologies are apparent within the LRR domain.

Discussion

Alleles at the *RPP2* locus are responsible for specifying recognition of *P. parasitica* (*At*) isolate Cala2 in *Arabidopsis* accession Col-0. Fine-scale mapping and mutational analysis were used to reveal that two TIR:NB:LRR genes, *RPP2A* and *RPP2B*, were required for effective resistance.

Although *RPP2A* encodes a protein that belongs to the TIR:NB:LRR class, it is of an unusual structure. It contains two TIR:NB regions, a potential CC domain and a DUF640 domain. The N-terminal TIR:NB region lacks associated ARC motifs. In contrast, the second TIR:NB region contains ARC motifs, but, surprisingly, lacks a pre-P- and a P-loop. A high level of identity (64%) exists between the proteins encoded by *RPP2A* and *RPP5* in the N-terminal TIR:NB domain; however, this is not the case for the second TIR:NB region. Meyers *et al*. (2002) proposed that the TIR:NB:LRR class of *R* genes could have evolved from a fusion between LRR and TIR proteins (TX), or TIR:NB proteins (TN). It is therefore possible that *RPP2A* and *RPP5* were formed by the fusion of a common TN gene with different LRR-containing proteins. For *RPP5*, this was a simple fusion, but in the case of *RPP2A*, the TN gene fused with a full-length TIR:NB:LRR gene. Alternatively, *RPP2A* may have been created by the fusion of the TIR:NB domain from *RPP5* (or its progenitor) and another TIR:NB:LRR-like gene. If the ARC, pre-P-loop and P-loop motifs are all essential for RPP2A function, they can clearly tolerate being separated within the protein.

Another intriguing feature of the RPP2A protein is the small LRR domain which consists of only seven repeats. Although proteins with as few as two or three LRRs, e.g. the Trk receptor kinase from humans and the TrkB receptor kinase from mouse, have been reported (reviewed by Kobe and Deisenhofer, 1994), such a small number of LRRs is unexpected for a plant disease-resistance gene (Jones and Jones, 1997). For example, the proteins encoded by *RPP5*, *N*, *L6* and *RPS4*, all of which belong to the TIR:NB:LRR subclass of *R* genes, are predicted to contain 21, 16, 27 and 15 LRRs, respectively (Gassman *et al*., 1999; Lawrence *et al*., 1995; Parker *et al*., 1997; Whitham *et al*., 1994). Only the genes in the *RPP1* cluster encode proteins containing as few as 10 LRRs (Botella *et al*., 1998).

The presence of a potential CC domain implies the possible formation of homo- or heterodimers. The role of the DUF640 domain is currently unknown. It has been detected simply as a conserved domain amongst certain plant genes in the PFAM database. *RPP2A* is the first DUF640-containing protein to which a function has been assigned. Other predicted proteins containing this motif are small peptides. In future, it will be interesting to learn if there are any common functional themes amongst these proteins.

RPP2B encodes an TIR:NB:LRR gene with a more classical structure. The N-terminal TIR domain and the following NB-ARC domain contain all the appropriate conserved

sequences (Figure 4). The LRR domain consists of fourteen 21–24-amino acid repeats, and is followed by a C-terminal domain of 282 amino acids. Mutations used to identify *RPP2B* lie in the NB-ARC region, the LRR region and a region of the C-terminal domain conserved amongst many TIR:NB:LRR proteins (motif 25/motif 8; Meyers *et al*., 2003). In RPP2B, this motif is positioned 10 amino acids from the last predicted LRR, and it itself shows sequence conservation reminiscent of an LRR. The most highly conserved component of the motif is (F/L)XFTNCF(K/N)L, which is similar to the LRR consensus of LXLXXCXXL. This suggests that this motif forms part of a degenerate LRR, but still plays a role in the function of RPP2B.

RPP2A and *RPP2B* are part of a *R*-gene cluster that contains two other TIR:NB:LRR genes. *At4g19520* codes for a TIR:NB:LRR protein containing an additional TIR domain at the C-terminus. *At4g19530* is a standard TIR:NB:LRR gene (Figure 1d). However, the lack of DNA homology between all four genes suggests that it is unlikely they arose as gene duplication events from a single progenitor. The *RPP2* locus may be analogous to the *Pto*-resistance complex in tomato. Although the *Pto* gene from tomato complements resistance to the bacterium *P. syringae* pv. *tomato* in susceptible plants (Martin *et al*., 1993), it has been shown that another gene, *Prf*, lying within the *Pto* cluster, is absolutely essential for effective resistance (Salmeron *et al*., 1996). Pto encodes a serine–threonine protein kinase, while Prf is an NB:LRR protein (Salmeron *et al*., 1996). It is thought that Pto and Prf act together to respond to *AvrPto*, which interacts directly with *Pto* (Scofield *et al*., 1996; Tang *et al*., 1996). It is therefore possible that products of all four genes at the *RPP2* locus interact as a resistance complex in a variety of different combinations. Alternatively, these proteins may interact to perform some function in plant growth and development that is targeted by the Cala2 *avr* gene product (ATR2). It is intriguing that a group of diverse but similar genes have evolved or been assembled at this locus.

Comparison of the LRR domains of TIR:NB:LRR genes by Meyers *et al*. (2003) revealed that there are highly conserved motifs (5, 14 and 15) at the beginning of the LRR domain. In common with three other TIR:NB:LRR genes in the Col-0 genome, RPP2A is exceptional in lacking all three of these motifs. Not only are these motifs missing, but the TIR:NB:LRR region does not conform to the standard structure. At4g36140 has the same modular structure as RPP2A with duplicated TIR:NB regions, At4g09360 lacks a TIR domain and At5g17970, classified as a possible pseudogene, lacks several conserved NB motifs (Meyers *et al*., 2003). Therefore, it is possible that these proteins may lack key motifs that enable them to function independently as *R* genes but are nevertheless required to effect specific recognition of particular pathogens and their variants. We have shown that RPP2A and RPP2B cooperate to effect resistance to Cala2, even though the latter is a TIR:NB:LRR protein that appears to contain all necessary conserved motifs to function independently. The implication is therefore that all three of these predicted genes may also function via a TIR:NB:LRR partner protein.

Inspection of the proteins encoded by the four genes at the *RPP2* cluster revealed a conserved structure (DLEGCTSL) within the third LRR. Interestingly, although this sequence is conserved absolutely between RPP2A and RPP2B, it is less well conserved within At4g19520 and At4g19530. Analysis of other TIR:NB:LRR genes reveal that there is sequence conservation in this LRR, but not to the degree observed between RPP2A and RPP2B. This absolute conservation is startling in the light of the lack of overall homology between RPP2A and RPP2B. This implies a significant functional role for this LRR in the cooperative action of these two proteins. This function could allow these proteins to interact directly or be related to a shared recognition capability of either a domain of the ATR2 protein or some other component plant protein.

Warren *et al*. (1998) identified a mutation (*RPS5.1*) in the third LRR of *RPS5*, a CC:NB:LRR class *R* gene that recognises *P. syringae* pv. *tomato* carrying the *avrPphB* gene. This mutation suppressed resistance specified by a range of bacterial and downy mildew *R* genes, including Cala2 resistance mediated by *RPP2*. A small but statistically significant increase in susceptibility to Cala2 was observed in the *RPS5.1* mutant. Warren *et al*. (1998) suggested that their observation could reflect an interaction with a common component of the signal transduction pathway, leading to the expression of resistance. An alternative explanation would be that the RPS5 protein is part of the same complex as the RPP2A/RPP2B TIR:NB:LRR proteins. Bittner-Eddy *et al*. (2000) described the *RPP13* gene, a CC:NB:LRR protein, that shows high sequence conservation to the third LRR of *RPS5.* However, resistance effected by *RPP13* was unaffected by the *RPS5.1* mutation (Bittner-Eddy and Beynon, 2001). Comparison of the conserved third LRR from RPP2A and RPP2B with that from RPS5 revealed no sequence homology. Hence, the pleiotropic effect of the *RPPS5.1* mutation neither relies on nor is associated with conserved sequences within the LRR domain. However, it is nevertheless remarkable that it is the third LRR that shows an absolute conservation of a subsequence between RPP2A and RPP2B, which does suggest functional significance for the third LRR, independent of the detection of the pathogen *avr* gene product.

RPP2B appears to have all the necessary components of a *R* gene, but to recognise Cala2, we have shown that it requires RPP2A. This implies some form of interaction between the gene products or the juxtaposition of these proteins as components of a complex. Two basic models exist for the interaction between the products of *R* and *avr* genes. The gene products could interact directly, which has been shown to occur in the case of Pto and AvrPto and

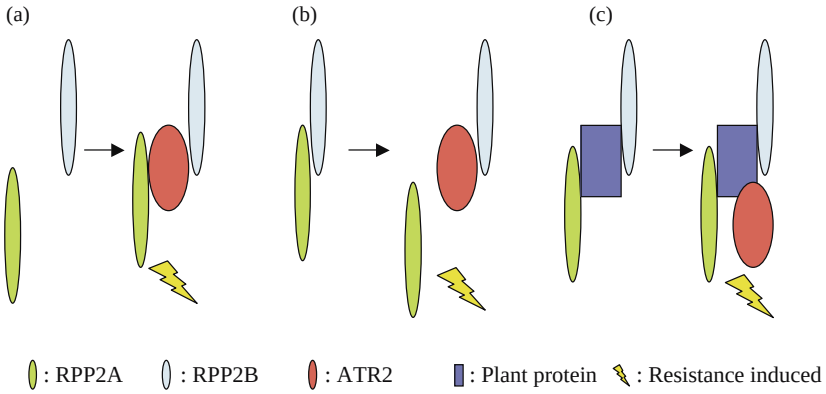

Figure 5. Models for the interaction of RPP2A and RPP2B with their avr target.
(a) The complementary avr protein (ATR2) recruits RPP2A to RPP2B and triggers the resistance response.
(b) A stable complex of RPP2A and RPP2B is disrupted by ATR2 triggering the resistance response.
(c) RPP2A and RPP2B are part of a complex stabilised by other plant proteins. ATR2 interacts with such intermediate proteins, may be resulting in release of RPP2A or RPP2B, to trigger the resistance response.
Three potential models for the RPP2 complex are proposed.

between Pi-ta and AVR-Pita (Jia *et al.*, 2000; Scofield *et al.*, 1996; Tang *et al.*, 1996). This model is shown in Figure 5(a,b), where direct interaction of the avr protein either recruits the *R* genes or de-stabilises their interaction. Moffet *et al.* (2002) demonstrated that the active domains of an R protein can be suppressed by intramolecular interactions, and the model in Figure 5(b) similarly shows suppression of the activation of resistance responses via intermolecular interactions between RPP2A and RPP2B.

It has, however, not proved possible to demonstrate direct interactions between R proteins and their partner AVR proteins in several pathosystems. This led Van der Biezen and Jones (1998) to propose that the *R*-gene protein could monitor (guard) another plant protein, and it is the interaction between that second protein and the *avr* gene product that results in the R protein, triggering a plant response to the pathogen. In this context, therefore, it is possible that RPP2A 'guards' RPP2B or vice versa, a model also consistent with Figure 5(b). When the Cala2 avr protein, ATR2, interacts with either the RPP2A or RPP2B target, the hypothesis is that the alternative gene product is released from the complex to initiate a disease-resistance response. However, it may also be that RPP2A and RPP2B both further guard a plant protein, which is itself the target of ATR2 (Figure 5c). In this model, interaction of ATR2 with the proposed third protein results in activation of the resistance response via RPP2A or RPP2B, or both. Finally, it may be that the detection of ATR2 requires the juxtaposition of two LRR domains, a model consistent with Figure 5(a).

The *RPP2* locus is an example of two TIR:NB:LRR genes apparently acting cooperatively to determine the recognition specificity to a single pathogen avirulence determinant. The discovery reported here implies that degenerate forms of TIR:NB:LRR genes may be maintained through evolution, specifically to generate the capability for novel recognition of pathogen gene products.

Experimental procedures

Arabidopsis *cultivation and pathogenicity tests*

The *Arabidopsis* accessions Col-0 and Nd-1, and the F_9 Col-0 × Nd-1 inbred lines (RIL), used to map *RPP2*, were as reported by Holub *et al.* (1994) and Bittner-Eddy *et al.* (1999). The conditions of plant cultivation, maintenance of *P. parasitica* (*At*) isolate Cala2 and pathogenicity tests were carried out as described by Holub *et al.* (1994).

Fine-scale mapping of the RPP2 *locus and development of a lambda clone contig spanning* RPP2A

Molecular markers *Agamous* and *B9*, and the phenotypic marker *RPP4* have been described elsewhere (Tör *et al.*, 1994). *B9* was converted into a CAPS marker (primers: 5′-CATCTGC-AACATCTTCCCCAG-3′ and 5′-CGTATCCGCATTTCTTCACTGC-3′; restriction enzyme *Aci*I) to facilitate the mapping of *RPP2* within the RIL population. Cosmid clone g3883 (Nam *et al.*, 1989) was provided by R. Schmidt and C. Dean (John Innes Institute, Norwich, UK) and converted into a CAPS marker (primers: 5′-TGTTTCAGAGTAGCCAATTC-3′ and 5′-CATCCATCAAACAAAC-TCC-3′; restriction enzyme *Pst*I). EST clone T46721 (Newman *et al.*, 1994) was developed as a RFLP marker, detecting a *Bgl*II restriction site polymorphism between Col-0 and Nd-1. This EST clone corresponds to At4g19920, which encodes a TIR:NB:LRR protein.

YAC clones were obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University, and the BAC clone F24J7 was obtained from RZPD (Berlin, Germany; Mozo *et al.*, 1998). The vectorette PCR amplification method, as described by Matallana *et al.* (1992), was used to generate end probes from YAC clones CIC4G1, CIC2G8 and yUP5C3. The end probes were either used as RFLP markers or, in the case of 4G1L, converted into a CAPS marker (primers: 5′-GTAACAC-

TATGGCTGTGGTAGAG-3′ and 5′-ACGAAATGTATTTCAATGTAA-TGT-3′; restriction enzyme *Apo*I).

Plant DNA was prepared from the RIL population using the CTAB extraction method (Ausubel *et al.*, 1994). Typically, 2–3 μg of plant DNA was digested with the appropriate restriction enzyme for RFLP analysis. For CAPS markers, 25 ng of plant DNA was used in a 25 μl PCR containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 200 mM each dNTP, 0.4 μM each primer and 1 unit Taq polymerase. A Perkin-Elmer 9700 thermocycler was used, and the amplification conditions were 1 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C, then a final extension at 72°C for 5 min. PCR products were purified by spun-column chromatography using Sepharose CL-6B (Amersham Pharmacia Biotech, Little Chalfont, UK), digested with the appropriate restriction enzyme and typically resolved on 2% agarose gels.

A lambda library, containing Col-0 genomic DNA, was used to create a contig of clones spanning the *RPP2A* gene. This library was created in the lambda vector GEM11 from size-fractionated digested Col-0 DNA (John Mulligan and Ronald Davis, Standford University, USA). Lambda clones were identified, and DNA was isolated using standard molecular techniques (Ausubel *et al.*, 1994). Restriction enzyme fingerprinting and Southern blot analysis, using radiolabelled DNA probes made from markers within the interval, was used to confirm and order the contig shown in Figure 1(b).

Mutant screening

Two Col-0 mutant populations were purchased from Lehle Seeds (Round Rock, TX, USA) and screened for susceptibility to Cala2. Approximately 14 000 M_2 generation seedlings were screened from both mutant populations. Mutants identified from the FN-treated population were given the prefix FN; mutants identified from the EMS-treated population were given the prefix EMS. Mutants were confirmed by testing progeny with Cala2. Mutants were also tested for defects in shared resistance mechanisms using seven other isolates avirulent on wild-type Col-0. Crosses using the mutants were performed as described by Bittner-Eddy *et al.* (1999). Three different crosses were performed to allow allelism tests and segregation analyses. Mutants were back-crossed with Col-0 using the wild-type plant as the pollen donor. The recovery of wild-type resistance in the resultant F_1 progeny was consistent with the recessive nature of the mutants. F_2 segregation analysis was used to determine the number of genes segregating for the observed mutant phenotypes. Mutants were also crossed with Nd-1 to determine those lines containing a mutation in a gene allelic to the susceptibility determinant present in Nd-1 (lack of *RPP2A* and *RPP2B* in this case). Selected lines from the three mutant classes (Classes 1, 2A and 2B) were also crossed within and between classes to determine those mutations that were allelic.

Sequencing RPP2A *and* RPP2B *cDNA and mutant alleles*

Plant genomic DNA was prepared using the CTAB extraction method (Ausubel *et al.*, 1994), and was used as template for sequencing selected *RPP2A* and *RPP2B* mutants. Primers for PCR amplification and sequencing were designed from published DNA sequence of BAC clone F24J7 (http://www.mips.biochem.mpg.de/) using PrimerSelect (DNASTAR, Madison, WI, USA). Primer sequences are available upon request. PCR products were purified by spun-column chromatography using Sepharose CL-6B (Amersham Pharmacia Biotech), checked for quality and quantity by agarose gel electrophoresis and then sequenced directly (approximately 200 ng per reaction) using big dye-terminator chemistries and an ABI PRISM 377 sequencer (Applied Biosystems). Sequence contigs were assembled using Auto-Assembler 2.0 (Applied Biosystems). Conceptual DNA translations and DNA/protein alignments were performed using the MAP-DRAW and MEGALIGN programs, respectively (DNASTAR).

Total RNA was isolated from 4-week-old Col-0 seedlings using an RNAeasy Mini kit (Qiagen, West Sussex, UK). First-strand cDNA (sscDNA) was produced from total RNA using the SMART PCR cDNA synthesis kit (CLONTECH, Hampshire, UK) according to the manufacturer's protocol. *RPP2A* and *RPP2B* cDNA was generated from sscDNA template using gene-specific PCR primer pairs. The 5′- and 3′ untranslated regions (UTRs) of *RPP2A* and *RPP2B* were defined from partial cDNA sequence deposited in the database (http://www.arabidopsis.org). Three or four PCR primer pairs, respectively, were used to generate overlapping *RPP2A* or *RPP2B* cDNA fragments for sequencing. At least one of the primers in each pair was designed to span a predicted intron/exon boundary (http://www.arabidopsis.org), thereby ensuring that any residual contaminating genomic DNA would not serve as a PCR template. cDNA fragments were sequenced using specific primers, and the resulting sequence was analysed, assembled, and conceptual DNA translations and DNA/protein alignments were performed as described above. Sequence of the PCR and sequencing primers are available upon request.

Bioinformatics

Sequence similarity searches of nucleotide and protein sequence databases at the National Center for Biotechnology Information (Bethesda, MD, USA) were performed using BLAST programs (Altschul *et al.*, 1997). The Conserved Domain Architecture Retrieval Tool (CDART; http://www.ncbi.nlm.nih.gov/BLAST/) was used to find conserved amino acid motifs within RPP2A, RPP2B, and the proteins encoded by the linked genes *Atg419520* and *Atg419530*.

Agrobacterium*-mediated transformation of* Arabidopsis

Recognition of *Peronospora parasitica 2A* and *RPP2B* were cloned into the binary cosmid vector pSLJ75515 (http://www.uea.ac.uk/nrp/jic/s3d_plas.htm) and mated into *Agrobacterium tumefaciens* strain GV3101. The clones were cultured under tetracycline (12.5 μg ml^{-1}) and gentamycin (25 μg ml^{-1}) selection. The whole plant vacuum infiltration method (Betchtold *et al.*, 1993) was used in all *Arabidopsis* transformation experiments. Transformants were selected by spraying seedlings 1–2 weeks after germination with L-phosphinothricin at 100 μg ml^{-1}.

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