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Four *Arabidopsis RPP* Loci Controlling Resistance to the *Noco2* Isolate of *Peronospora parasitica* Map to Regions Known to Contain Other *RPP* Recognition Specificities

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Interactions between *Arabidopsis thaliana* and the downy mildew fungus *Peronospora parasitica* provide a model system to study the genetic and molecular basis of plant-pathogen recognition. With the use of the *Noco2* isolate of *P. parasitica*, the reaction phenotypes of 46 accessions of *Arabidopsis* were examined and 31 accessions exhibited resistance. Resistance phenotypes examined ranged from distinct necrotic pits or flecks to a weak necrosis accompanied by late and sparse fungal sporulation. Segregating populations generated from crosses between the susceptible accession Col-0 and the resistant accessions Ws-0, Pr-0, Oy-0, Po-1, Bch-1, Ge-1, Di-1, Ji-1, and Te-0 were also screened with *Noco2*. The genetic data were consistent with the presence of single resistance (*RPP*) loci in all of these accessions except Oy-0, in which resistance was inherited as a digenic trait. As a first step to molecular cloning, the map positions of four resistance loci were determined. These have been designated *RPP14.1* from Ws-0, *RPP14.2* from Pr-0, and *RPP14.3* and *RPP5.2* from Oy-0. *RPP14.1* was mapped to a 3.2-cM interval on chromosome 3 that is linked to a region between the markers *Gl-1* and m249 known to contain other *P. parasitica* resistance specificities. *RPP14.2* from Pr-0 and *RPP14.3* from Oy-0 were also positioned in this interval. Moreover, *RPP14.1* and *RPP14.2* showed linkage of <0.05 cM, suggesting possible allelism. The second *RPP* locus from Oy-0, *RPP5.2,* was located on chromosome 4 and exhibited strong linkage (<2 cM) to *RRP5.1*, a locus previously identified in the *Arabidopsis* accession Landsberg-*erecta*. The results reinforce evidence for *RPP* gene clustering in the *Arabidopsis* genome and provide new targets for cloning and examination of *RPP* gene structure, function, allelic variation, and organization within defined loci.

Additional keyword: disease.

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Genes conferring resistance to plant pathogens ("*R*" genes) have long been used to breed crop varieties less susceptible to yield losses caused by disease. The simplest model to explain the resistance response or "incompatibility" in most plant-pathogen combinations is that specific recognition of the parasite carrying an avirulence gene (*Avr* gene) is controlled by the plant carrying a corresponding *R* gene. This "gene-for-gene" relationship was first established for the interaction between flax and the flax rust fungus (*Melampsora lini*) (Flor 1955, 1971) and it has been found to hold for many other plant-parasite interactions (Crute 1985; Keen 1990).

Recently, several laboratories have been successful in the molecular cloning of *R* genes, providing essential information for an understanding of their mode of action. *R* genes have been isolated from cultivated crops—tomato (Martin et al. 1993, Jones et al. 1994), tobacco (Whitham et al. 1994), and flax (Lawrence et al. 1995)—and from the cruciferous plant *Arabidopsis thaliana* (L.) Heynh. (Bent et al. 1994; Mindrinos et al. 1994; Grant et al. 1995). Comparison of the sequences of these genes indicates that they share common structural motifs (Staskawicz et al. 1995), supporting the hypothesis that they have a common function and origin.

Arabidopsis has become a widely used model plant in molecular plant pathology (Dangl 1993). It is susceptible to viral (Li and Simon 1990), bacterial (Simpson and Johnson 1990; Tsuji et al. 1991; Davis et al. 1991; Parker et al. 1993a), fungal (Koch and Slusarenko 1990; Dangl et al. 1992), and nematode-induced (Sijmons et al. 1991) diseases. A particularly useful model for analyzing the molecular basis of recognitional specificity in *Arabidopsis* is provided by its interaction with the oomycete pathogen, *Peronospora parasitica* (Pers.:Fr.) Fr. This obligate biotroph causes downy mildew in the Cruciferae (Channon 1981) and certain *P. parasitica* isolates (or "pathotypes") are able to infect and complete their life cycles in *Arabidopsis* (Koch and Slusarenko 1990; Crute et al. 1993). In this interaction considerable genetic diversity has been uncovered in both the plant host and the pathogen and this is accompanied by a wide range of phenotypic responses (Holub et al. 1994). At least 19 resistance loci (*RPP* loci) controlling recognition of individual fungal races by different *Arabidopsis* accession lines have been inferred (Crute et al. 1994b). Some of these resistance loci have been posi-

tioned on the *Arabidopsis* linkage map (Parker et al. 1993b; Tör et al. 1994; Crute et al. 1994a, 1994b) and evidence of *RPP* gene clustering in the genome has been presented (Crute et al. 1994a).

In this study, our aim was to extend our knowledge about different *Arabidopsis* loci involved in the recognition of *Peronospora parasitica*. We focused on interactions with the *Noco2* isolate, which has been shown previously to be recognized by the *RPP5* locus in the *Arabidopsis* accession Landsberg-*erecta* (La-*er*) (Parker et al. 1993b). Identification, mapping, and cloning of *RPP* genes in this model system will enable us to study their organization within defined loci and analyze non-allelic interactions between the specific *RPP* genes involved.

We screened 46 *Arabidopsis* accessions with *P. parasitica* race *Noco2*. The majority were able to impede fungal development and were therefore classed as resistant. Considerable variation in the resistance phenotypes of these accessions was observed. For nine of them, the genetic inheritance of the resistance has been studied. As a prelude to positional cloning, we have defined the map locations of the four *RPP* loci in accessions Ws-0, Pr-0, and Oy-0. They map to regions known to contain other *RPP* specificities, reinforcing the notion that *RPP* genes are clustered in the *Arabidopsis* genome.

RESULTS

Identification of *Arabidopsis* accessions resistant to race *Noco2* of *Peronospora parasitica*.

Individual *Arabidopsis* accessions were screened for resistance or susceptibility to *P. parasitica* by means of the cotyledon assay and a standard fungal inoculum, as described previously (Dangl et al. 1992; Parker et al. 1993b). Where possible, we have classified the reaction phenotypes of the different *Arabidopsis* accessions to race *Noco2* according to the system of Holub et al. (1994). Forty-six *Arabidopsis* accessions were tested by inoculating 12 to 15 seedlings of each with a suspension of fresh *Noco2* conidia and their reactions were compared with the "EH" ("early and heavy sporulation") phenotype (Holub et al. 1994) of the *Noco2*-susceptible accession Col-0, characterized in a previous study (Parker et al. 1993b). The accession names, their geographical origin, and their phenotypic responses to the fungus are shown in Table 1. In this first phase of the study, the interaction phenotypes were classified into only three groups: "N" for "necrosis" (which includes the PN, CN, FN, and FR phenotypes), "FDL," and EH (see Materials and Methods for an explanation of designations). The majority (31) of the tested accessions showed some degree of resistance to *Noco2*. Fifteen exhibited an EH phenotype and were therefore classed as fully susceptible to *Noco2*. No obvious correlation was apparent between the geographical origin of an accession and its phenotypic response.

A set of nine accessions exhibiting the resistant N or FDL phenotypes was selected for further examination. These accessions were Ws-0, Pr-0, Oy-0, Po-1, Bch-1, Ge-1, Di-1, Ji-1, and Te-0. Prior to genetic investigation, their phenotypes were re-evaluated according to the full five-class scheme of Holub et al. (1994). The results are shown in Table 2 and Figure 1. The predominant resistant reaction was the PN phenotype, which was found in six of the nine accessions. In these seedlings, necrotic areas were visible 3 days after inoculation. The necrosis became more distinct after 4 to 5 days (see Figure 1B and C) and was easily distinguished from the FN (necrotic flecks) phenotypic class, exemplified by Oy-0 (Fig. 1D). It was noted that the "pitting" in accession Pr-0 developed differently than that observed in Ws-0 seedlings. Inoculated Pr-0 cotyledons became more chlorotic after 5 to 7 days whereas the pitting in Ws-0 remained discrete (compare Figure 1B and C). Microscopic observation of lactophenol-trypan blue-stained material showed that in both Ws-0 (Fig. 1b) and Pr-0 (Fig. 1c) cotyledons, necrosis of predominantly mesophyll cells occurred at sites of attempted fungal ingress. In both accession lines, fungal growth was completely prevented beyond 10 to 15 necrotic plant cells. Oy-0 cotyledons chal-

Table 1. Origins and reaction phenotypes of 46 *Arabidopsis* accessions challenged with the *Noco2* isolate of *Peronospora parasitica*

Accession	Geographical origin	Phenotype[a]
Aa-0	Aua/Rhön (Germany)	EH
Abd-0	Aberdeen (UK)	N
Ag-0	Argentat (France)	FDL
Ak-1	Achkarren/Freiburg (Germany)	FDL
An-1	Antwerpen (Belgium)	EH
Ang-1	Angleur (Belgium)	FDL
Bay-0	Bayreuth (Germany)	FDL
Bch-1	Büchen/Lauenburg (Germany)	FDL
Bd-0	Berlin/Dahlem (Germany)	FDL
Be-0	Bensheim/Bergstr. (Germany)	EH
Bl-1	Bologna (Italy)	N
Bla-1	Blanes/Gerona (Spain)	EH
Bs-1	Basel (Switzerland)	EH
Bsch-0	Buchschlag/FFM (Germany)	N
Bu-0	Burghaun/Rhön (Germany)	FDL
Bur-0	Burren (Eire)	EH
Ca-0	Camberg/Taunus (Germany)	EH
Cal-0	Calver (UK)	EH
Cen-0	Caen (France)	EH
Chi-0	Chisdra (former USSR)	N
Co-1	Coimbra (Portugal)	N
Condara	Khurmatov (Tadjikistan)	EH
Di-1	Dijon (France)	N
Ema-1	East-Malling (UK)	N
Ge-1	Geneva (Switzerland)	N
Je54	Relichova (former Czechoslovakia)	EH
Ji-1	Norwich (UK)	N
H-55	Relichova (former Czechoslovakia)	EH
Kas-1	Kashmir (India)	N
Kn-0	Kaunas (Lithunia)	N
Li-6	Limburg (Germany)	EH
Mh-0	Mühlen (Poland)	FDL
Ms-0	Moscow (Russia)	N
Mt-0	Martuba/Cyrenaika (Lybia)	N
Nd-0	Niederzenz (Germany)	EH
Oy-0	Oystese (Norway)	N
Po-1	Poppelsdorf (Germany)	N
Pr-0	Frankfurt/Praunheim (Germany)	N
RLD1	Koorneef goup (The Netherlands)	FDL
Rld-2	Rschew (Russia)	FDL
S96	Koorneef goup (The Netherlands)	N
Se-0	San Eleno (Spain)	EH
Te-0	Tenela (Finland)	N
Tsu-1	Tsu (Japan)	FDL
Wei-1	Weiningen (Switzerland)	N
Ws-0	Vasljevici/Dnjepr (former USSR)	N

[a] Phenotypes were assessed from 3 to 7 days after inoculation of 9-day-old seedlings with *Noco2* conidia. "N" (necrotic) phenotype = classes "PN" (necrotic pits), "CN" (necrotic cavities), and "FR" (necrotic flecks with rare sporulation); "FDL" = necrotic flecks with delayed and light sporulation; "EH" = early and heavy sporulation (see Materials and Methods and Holub et al. 1994).

lenged with *Noco2* exhibited an FN phenotype (Fig. 1D). Fungal growth did not spread beyond an area of three or four necrotic mesophyll cells (Fig. 1d), and so the light "flecking" appeared to be due to a very efficient containment of fungal development by the plant. One example of the FDL resistance response, Bch-1 (Table 2), was examined in more detail. In these plants, *Noco2* asexual sporulation was late (not observed at 3 days) and sparse (Fig. 1E), and in occasional seedlings no sporophores were observed macroscopically. Microscopic examination showed that mycelial development was not severely restricted (Fig. 1e) and was associated with sporadic plant cell necrosis. Therefore, the FDL phenotype of Bch-1 clearly represented a weaker resistance reaction than the PN, CN, and FN classes. One accession line, Di-1, produced an FR response

Table 2. Phenotypic characterization of nine resistant *Arabidopsis* accessions and corresponding F_1 seedlings from a cross with the susceptible accession Col-0, after inoculation with *Peronospora parasitica Noco2* conidia

	R accession								
	Ws-0	Pr-0	Oy-0	Po-1	Bch-1	Ge-1	Di-1	Ji-1	Te-0
	Phenotype[a]								
Individuals exhibiting an FDL reaction	**PN**	**PN**	**FN**	**PN**	**FDL**	**PN**	**FR**	**PN**	**PN**
Parent	0/10	0/11	0/12	0/10	7/10	0/9	1/9	0/12	0/11
F1	5/12	3/7	0/11	3/12	10/14	3/18	2/14	0/16	1/19

[a] Phenotypes were assessed from 3 to 7 days after inoculation of 9 day old seedlings with *Noco2* conidia. Phenotypic classes are as described in Table 1.

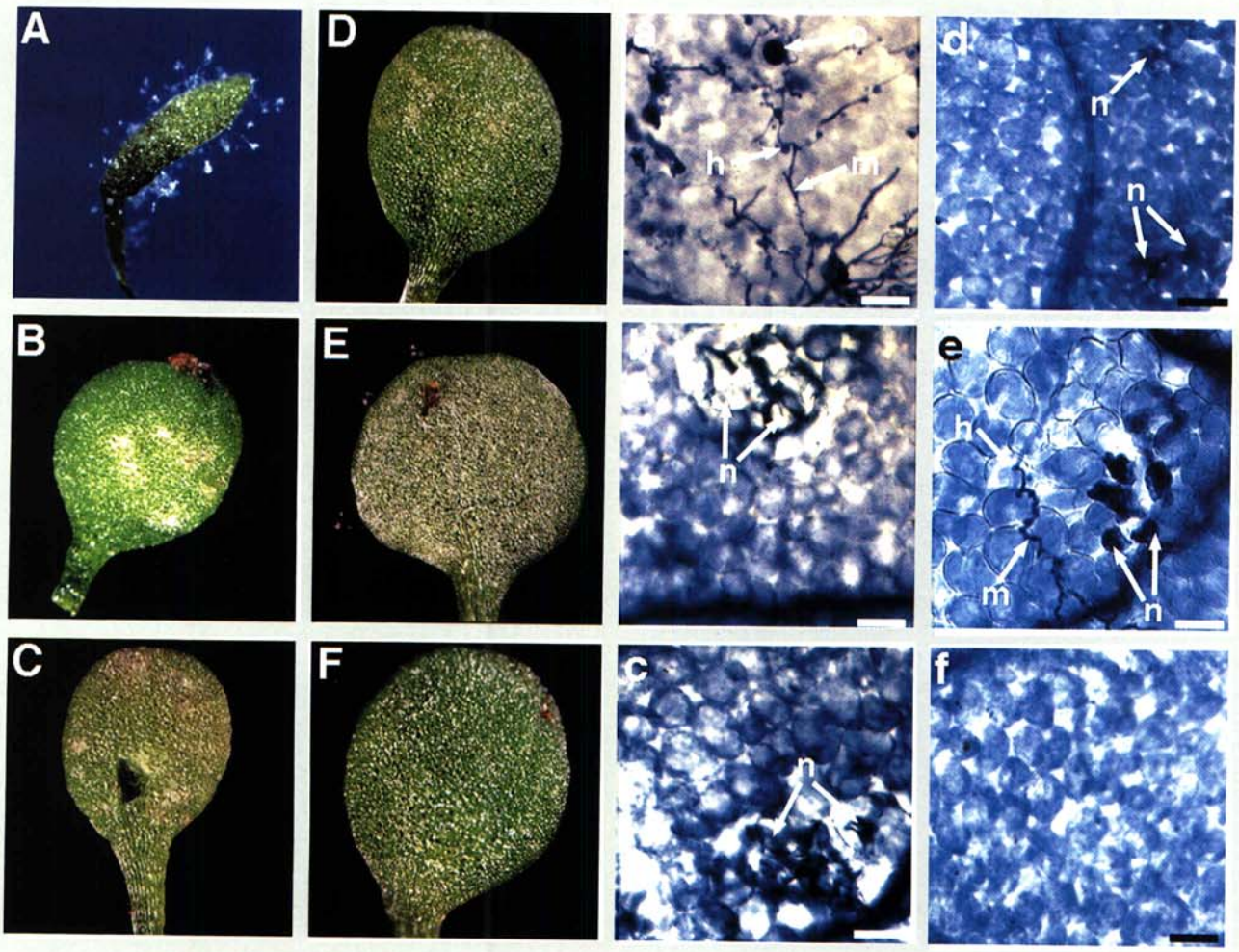

Fig. 1. Reaction phenotypes of cotyledons from different accessions of *Arabidopsis thaliana* 6 days after inoculation with *Peronospora parasitica* isolate *Noco2*. Phenotypes were monitored macroscopically (**A** to **F**) and microscopically by observing lactophenol-trypan blue-stained cotyledons (**a** to **f**). m = mycelium; h = haustoria; o = oospore; n = plant cell necrosis; bar = 50 μm. **A,** Profuse asexual *Noco2* sporulation in Col-0 associated with (**a**), extensive mycelial development and the production of oospores. **B,** Resistant PN (necrotic pitting) phenotype of Ws-0, with (**b**), extensive areas of necrotic mesophyll cells. **C,** Resistant PN phenotype of Pr-0, exhibiting chlorosis of the cotyledon and (**c**), extensive areas of necrotic mesophyll cells. **D,** Resistant FN (necrotic flecking) phenotype of Oy-0, associated with (**d**), discrete areas of mesophyll cell necrosis surrounding points of attempted *Noco2* ingress. **E,** Resistant FDL (necrotic flecking with delayed and light sporulation) phenotype of Bch-1 associated with extensive mycelial development (**e**). **F,** Uninoculated Col-0 cotyledon with (**f**), no areas of mycelial growth or host cell necrosis.

to *Noco2* according to the classification of Holub et al. (1994) (Table 2). Microscopically this was not readily distinguishable from FDL plants except in the average numbers of sporophores produced in five pairs of cotyledons of each accession examined (results not shown). The degree of fungal ingress and extent of plant cell necrosis were similar in Bch-1 and Di-1.

In summary, a range of phenotypes was revealed in different *Arabidopsis* accessions after inoculation with *Noco2* and it was of interest to examine the inheritance of the resistance in these selected plant lines.

Genetic analysis of resistance to *Noco2* in accessions Ws-0, Pr-0, Oy-0, Po-1, Bch-1, Ge-1, Di-1, Ji-1, and Te-0.

F_1 plants and segregating F_2 populations generated between the susceptible line Col-0 (or Col-0 containing the recessive genetic marker *glabrous* [*gl-1*]) and each of the selected resistant accessions were inoculated with *Noco2* to investigate the genetic control of the resistance response.

The most detailed study was performed on Ws-0, since this particular line had been used in a large-scale T-DNA insertional mutagenesis program (Feldman 1992) and therefore offered a potential opportunity to isolate an insertional mutant allele of an identified *RPP* gene. Macroscopic analysis of 195 inoculated Col-*gl* × Ws-0 F2 seedlings showed that 46 gave the PN phenotype and 39 an EH phenotype. An intermediate phenotype reminiscent of the FR and FDL classes was exhibited by 102 seedlings. Eight plants gave no obvious phenotype and were presumed to be inoculation "escapes." The data are consistent with presence of a single semidominant resistant locus in Ws-0 (see Table 3). This was also suggested by the intermediate (FDL) phenotype observed in a proportion (5/12) of Col-*gl* × Ws-0 F1 seedlings in response to *Noco2* inoculation, as shown in Table 2.

The remaining segregating populations were scored by counting the number of EH individuals and the number of individuals exhibiting any degree of resistance (PN, FN, FR, and FDL phenotypes). The results of these analyses are summarized in Table 3. The 3:1 segregation of resistance/susceptibility to *Noco2* in all F_2 populations, except that derived from Oy-0, was consistent with the presence of a single dominant or semidominant *RPP* locus in each resistant parent. F_1 seedlings for each parental × Col-0 combination were also inoculated with *Noco2* and scored for the presence of an intermediate phenotype compared with resistant and susceptible (Col-0) parents. As shown in Table 2, an intermediate response, manifested macroscopically as an FDL phenotype, was observed in a proportion of F_1 seedlings from Pr-0, Po-1, and Ge-1, as well as from Ws-0 (described above). This suggests that in the cross with the susceptible line Col-0 the *RPP* gene action of these resistant parents is semidominant. In contrast, F_1 seedlings from Ji-1 and Te-0 were phenotypically similar to their wild-type parents and were therefore not obviously semidominant. In the case of Bch-1 and Di-1, which exhibit FDL and FR phenotypes, respectively, there was no obvious increase in fungal development in the F_1 seedlings, making it difficult to interpret the degree of dominance without further analysis.

Two different segregating populations were available for the genetic analysis of the FN type of resistance to *Noco2* in accession Oy-0: a Col-0 × Oy-0 F_2 population produced for this study, and F_8 generation RIs made between the susceptible accession Nd-0 and Oy-0 (a gift from Thomas Debener and Jeff Dangl, Cologne, Germany). Segregation data derived from both populations suggested the presence of at least two unlinked *RPP* loci in Oy-0, as shown in Table 3. In the F_2 analysis, the resistance/susceptibility ratios did not deviate significantly from a 15:1 segregation and were not consistent with the presence of three unlinked *RPP* loci ($\chi^2 = 20.6$ at $P = 0.05$). All resistant F_2 seedlings exhibited the FN phenotype in response to *Noco2*, suggesting that each *RPP* locus was able to confer a FN response independently of the second locus. In the analysis of 98 Oy-0 × Nd-0 RIs, 12 to 16 individuals of each RI line were inoculated with *Noco2*. The resistance/susceptibility ratio was 80:18 (≈ 5:1). Analysis (χ^2) of the data showed that it was consistent with both two (3:1 ratio) or three (7:1 ratio) *RPP* loci controlling resistance to *Noco2* (see Table 3). Interestingly, seven of the 18 *Noco2*-susceptible RI lines consistently exhibited delayed and reduced sporulation compared with the remaining 11 EH susceptibles. However, this lack of full susceptibility was not as strong phenotypically as the FDL phenotype and necrotic flecks were not observed. It is possible, therefore, that an extremely weak resistance locus is segregating in these lines. Certainly the data point to the existence of at least two independent loci mediating a phenotypically "strong" (FN) resistance to *Noco2*.

Map location of *RPP14* in Ws-0.

Holub et al. (1994) reported that resistance in Ws-0 to *Noks1*, a culture generated from a single *Noco2* oospore, cosegregated with resistance to other *P. parasitica* isolates in F_8 RIs made from a cross between Ws-0 and W100 (La-*er* background). These resistance loci were designated *RPP1* (controlling resistance to isolate *Emoy2*) and *RPP10* (resistance to isolate *Cala2*) and they mapped to an interval of approximately 10.5 cM defined by the markers OPC12 and

Table 3. Segregation analysis of resistance and suceptibility in F_2 populations made from *Noco2*-resistant *Arabidopsis* accessions and the susceptible accession Col-0 (or Col-*gl*), and in recombinant inbred lines derived from the *Noco2*-resistant accession Oy-0 and the susceptible accession Nd-0[a]

Segregating population	Resistant[b]	Susceptible	R/S (expected ratio)	χ^2 (1 df)[c]
F_2 Col-*gl* × Ws-0[d]	148	39	3:1	1.82
F_2 Col-*gl* × Pr-0	148	42	3:1	1.02
F_2 Col-0 × Po-1	65	18	3:1	0.56
F_2 Col-0 × Bch-1	101	37	3:1	0.35
F_2 Col-0 × Ge-1	117	39	3:1	0.00
F_2 Col-0 × Di-1	43	13	3:1	0.09
F_2 Col-0 × Ji-1	112	28	3:1	1.86
F_2 Col-0 × Te-0	102	26	3:1	2.66
F_2 Col-0 × Oy-0	243	13	15:1	0.60
RIs Oy-0 × Nd-0	80	18	3:1	1.98
			7:1	3.42

[a] Nine-day-old seedlings were assessed 3 to 7 days after inoculation with *Noco2* conidia.

[b] Resistance was scored for cotyledons exhibiting any degree of necrosis (PN, FN, FR, and FDL phenotypes). Susceptibility was scored for cotyledons showing an EH phenotype equivalent to the control Col-0 seedlings. Phenotypic classes are described in Table 1 and Materials and Methods.

[c] Chi-square analysis showed that the observed segregation resistance/susceptibility (R/S) ratios did not deviate significantly from the indicated expected ratios at $P = 0.05$.

[d] Col-*gl* is Col-0 carrying the recessive genetic marker *glabrous1*.

m249 on chromosome 3 (Holub et al. 1994; Tör et al. 1994). In the present study, analysis of 60 *Noco2*-susceptible F_2 individuals (or corresponding F_3 families) from the Col-*gl* × Ws-0 cross (Table 3) confirmed linkage of the *Noco2*-recognition locus, which we have designated *RPP14*, to the marker *Gl-1* (8% recombination). A further 30 F_3 families, consisting of 17 homozygous *Noco2*-susceptibles and 13 families segregating for *RPP14*, were added to the initial population to give a mapping population of 90 F_2 plants. Polymorphic molecular markers in the region of *Gl-1* and m249 were then used to refine the position of *RPP14*. Five recombinant chromosomes placed the marker mi358 4.2 cM north of *RPP14* and one recombinant chromosome placed the marker pAT3-89.1 1.4 cM south. Additional recombinants in the interval between *RPP14* and *Gl-1* (*RPP14/- gl-1 /gl-1*and *RPP14/RPP14 Gl-1/-* plants) were also selected phenotypically. All F_3 families that had a recombination event in the *Gl-1* to m249 interval were then tested with the marker ve021 and this was placed 1.8 cM north of *RPP14*. Therefore, *RPP14* was positioned in a 3.2-cM interval between the markers ve021 and pAT3-89.1 on chromosome 3 as shown in Figure 2A. In accordance with the scheme for *RPP* gene nomenclature set out by Crute et al. (1994a), in which a newly identified *RPP* recognition specificity directed to a particular *P. parasitica* isolate is given a new number, we have designated this locus *RPP14.1*

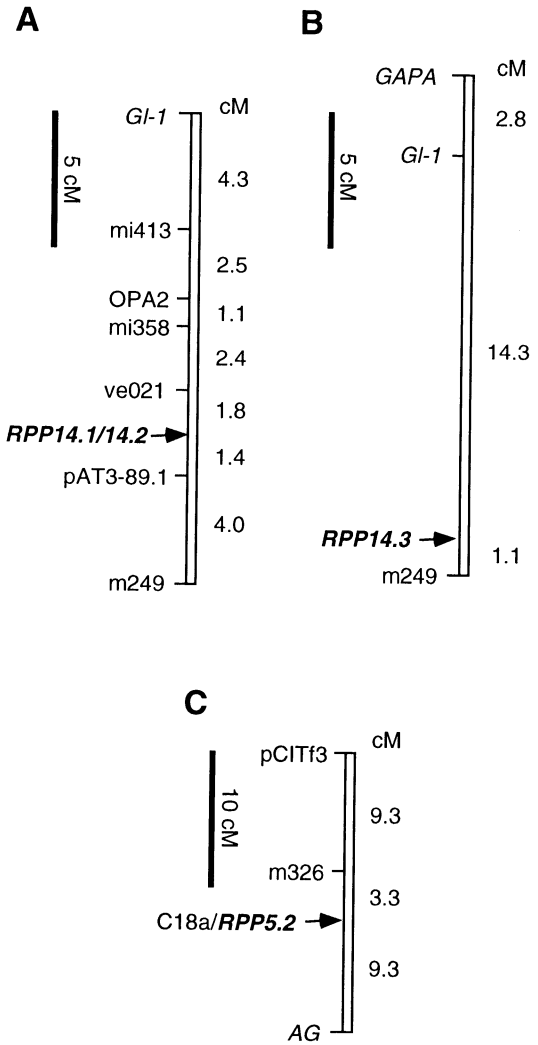

Fig. 2. A, Linkage map of part of *Arabidopsis* chromosome 3 showing the location of *RPP14.1* from Ws-0 and *RPP14.2* from Pr-0 relative to molecular markers, based on the segregation analysis of Col-*gl* × Ws-0 and Col-*gl* × Pr-0 F_2 populations, and analysis of test cross progeny derived from a Col-*gl* × *RPP14.1/RPP14.2* cross. **B,** Map position on chromosome 3 of *RPP14.3* from Oy-0 based on the segregation analysis of 41 Col-0 × Oy-0 *Noco2*-susceptible F_2 seedlings. **C,** Map position on chromosome 4 of *RPP5.2* from Oy-0 from the analysis of the same population as in **B**.

Map location of the *Noco2*-resistance in Pr-0.

Phenotypic analysis of F_2 seedlings generated from a cross between Col-*gl* and Pr-0 showed that resistance to *Noco2* was inherited as a monogenic trait (see Table 3). Forty-eight *Noco2*-susceptible (EH) F_2 individuals were selected as the initial mapping population. Scoring of the *glabrous* phenotype in these plants quickly established that the Pr-0 resistance was linked to *Gl-1*, as with *RPP14.1*. Seven plants had wild-type trichomes, indicating a recombination frequency of approximately 7%. Molecular markers north and south of *Gl-1* were tested for polymorphisms between Pr-0 and Col-*gl* DNA (see Materials and Methods). Two suitable markers, mi358 and GAP-A, allowed us to place the Pr-0 resistance locus south of *Gl-1*, indicating that its position was close to *RPP14.1*, and certainly south of mi358 (see Figure 2A).

Test for allelism between the Ws-0 and Pr-0 resistance loci.

In order to test more rigorously the relative positions of the Ws-0 resistance locus *RPP14.1* and the newly identified Pr-0 locus, F_1 progeny were generated between Ws-0 and Pr-0 and these individuals were test crossed to the *Noco2*-susceptible accession Col-*gl*. This experiment was designed to identify any recombination events in the short but as yet undefined interval between the two loci, as these would give rise to *Noco2*-susceptible progeny. No susceptible individuals were obtained after inoculation of 2,240 seedlings. Therefore, the Ws-0 and Pr-0 resistance loci were separated by a genetic distance of less than 0.05 cM. This is illustrated in Figure 2A and the Pr-0 locus is accordingly designated *RPP14.2*.

Mapping the *Noco2*-resistance loci in Oy-0.

The segregation data derived from Oy-0 × Nd-0 RIs and from Col-0 × Oy-0 F_2s (Table 3) were consistent with the presence of at least two unlinked *RPP* loci in Oy-0 controlling resistance to *Noco2*. Mapping of these loci on the RIs would simplify the analysis since each RI line would be essentially homozygous for parental DNA at a particular locus (Burr and Burr 1991), avoiding ambiguity in scoring heterozygotes of potentially semidominant loci. However, a very low level of polymorphism between selected molecular markers in Oy-0 and Nd-0 was encountered, and mapping on this population was abandoned. A higher level of polymorphism was found between Oy-0 and Col-0, and 41 *Noco2*-susceptible F_2 plants derived from this cross (see Table 3) were used as an initial mapping population. CAPS (Konieczy and Ausubel 1993) and microsatellite DNA markers (Bell and Ecker 1994) were used to assess linkage, as shown in Table 4. Two markers were identified that exhibited clear linkage to the Col-0 susceptibility. These were *Gl-1* on chromosome 3 (14% recombination), suggesting proximity to the *RPP* loci previously identified in the *Gl-1* to m249 interval (*RPP1* and *RPP10*, Tör et al. 1994;

RPP14, the present study), and *AG* on chromosome 4 (9% recombination), again pointing to possible linkage to several *RPP* specificities identified in the region of *Agamous* (*RPP5*, Parker et al. 1993b; *RPP2* and *RPP4*, Tör et al. 1994). Recombination frequencies of no less than 40% were observed with molecular markers on chromosomes 1 and 5 (Table 4), indicating an absence of linkage on these chromosomes. No suitable polymorphic marker was found on chromosome 2. Polymorphisms were sought for molecular markers north and south of these two linkage points in order to confirm and consolidate the mapping positions (see Table 4). The map positions of the two RPP loci derived from this analysis are shown in Figure 2B and C. One *RPP* locus in Oy-0 has been designated *RPP14.3*, since its position derived from this F_2 population is consistent with it being part of the *RPP14* cluster, lying 1.1 cM north of m249. The second *RPP* locus is designated *RPP5.2*, because it was found to cosegregate (analysis of 62 gametes) with a CAPS marker, C18a, that is known from a previous study to map less than 0.2 cM from *RPP5.1* (Parker et al. 1993b). This would place *RPP5.2* within 2 cM of *RPP5.1*, but also in the region of *RPP4* (Tör et al. 1994). The designation of *RPP14.3* and *RPP5.2* loci is tentative since it is based on a limited mapping population size. However, the analysis identifies areas of the *Arabidopsis* genome already known to contain genetically defined *RPP* specificities.

DISCUSSION

Genetic analyses of different *Arabidopsis* accessions with a range of *P. parasitica* isolates have revealed the presence of phenotypically distinct *RPP* loci controlling resistance to this oomycete pathogen (Crute et al. 1993; Parker et al. 1993b; Holub et al. 1994). A great potential exists, therefore, to exploit the genetic and molecular amenability of *Arabidopsis* to isolate these *RPP* genes, to identify other interacting signaling components involved in plant-pathogen recognition, and to determine the mechanisms responsible for the resistance phenotypes.

We previously identified a locus, *RPP5* in La-*er*, that recognizes the *P. parasitica* isolate *Noco2*. This was mapped to a 1.2-cM interval on the upper arm of chromosome 4 (Parker et al. 1993b). The aim of the present study was to identify new *RPP* recognition specificities controlling resistance to *Noco2* and to target some of these for cloning and subsequent molecular analysis. The genetic analysis of segregating populations derived from nine different *Noco2*-resistant parents showed that in all of them resistance was inherited as a monogenic trait, except in one, Oy-0, which possesses at least two distinct *RPP* loci. Within the scope of the mapping analyses carried out for the monogenic resistances in Ws-0 and Pr-0 and the digenic resistance in Oy-0, it was apparent that the targeted loci were distributed in only two locations. These areas of the genome are known to contain *RPP* genes; *RPP5* (Parker et al. 1993b) and *RPP4* (Tör et al. 1994) in the interval between m226 and *Agamous* on chromosome 4, and a cluster of *RPP* recognition specificities in the *Gl-1* to m249 interval on chromosome 3 (Tör et al. 1994). We would predict that *Noco2* possesses at least two *avr* loci that incite a phenotypically distinct resistance response in *Arabidopsis*. These would accordingly be designated *AvrPp5* and *AvrPp14*. Our data reinforce preliminary evidence that *RPP* recognition specificities are clustered in the *Arabidopsis* genome (Crute et al. 1994a, 1994b) and this parallels what is known to exist for resistance genes in several crop plant species, such as the *Rp1* rust resistance locus in maize (Saxena and Hooker 1968; Hulbert and Bennetzen 1991), the *L* and *M* rust resistance loci in flax (Ellis et al. 1988; Lawrence et al. 1995), the *Dm* genes controlling resistance to downy mildew in lettuce (Hulbert and Michelmore 1985), and the *Cf* resistance genes of tomato (Jones et al. 1993). Indeed, there is growing evidence that genes controlling resistance to different pathogen species with strikingly different modes of attack are clustered in plant genomes (Pryor 1987; Kesseli et al. 1993). This supports the hypothesis that many recognition specificities are members of multigene families that have arisen from a common ancestral gene and are therefore highly related (Pryor and Ellis 1993; Staskawicz et al. 1995). In this context, the multiple *RPP* specificities identified within the *RPP1/10/14* cluster are of great interest. Cloning one *RPP* gene could expedite the isolation of related resistance genes, and we could begin to examine the evolution of these genes and uncover the molecular basis to allelic variation. The mapping of *RPP14.1* in Ws-0 to a 3.2-cM interval described in the present study is an essential step toward this goal and should ease the cloning of the tightly

Table 4. Analysis of genetic recombination between loci in Oy-0 controlling resistance to *Peronospora parasitica* isolate *Noco2* and *Arabidopsis* molecular markers

Marker	Position/ chromosome[a]	Total chromosomes	Recombinant chromosomes	Recombination frequency (%)
nga280	115/I	74	39	53
ATHGENEA	120/I	68	38	56
ATHATPASE	154/I	74	43	58
ATHCHIB	19/III	78	32	41
GAPA[b]	56/III	78	13	17
Gl-1	65/III	74	10	14
m249[b]	80/III	58	2	3
pCITf3[b]	39/IV	60	9	15
m326[b]	62/IV	62	2	3
C18a[b]	67/IV	62	0	0
AG	73/IV	76	7	9
nga76	76/V	80	32	40
nga129	119/V	80	35	44

[a] Marker positions in cM are from the recombinant inbred map released by C. Lister and C. Dean on the Internet (June 1995). No suitable polymorphic marker was found on chromosome II.

[b] Markers indicated were used for refinement of linkage map based on initial linkage to Gl-1 and AG.

linked loci *RPP14.2* in Pr-0 and *RPP14.3* in Oy-0. The numerical classification of *RPP* resistance specificities used here conforms to the scheme adopted by Crute et al. (1994a) in which an *RPP* recognition specificity to a defined *P. parasitica* isolate (or pathotype) identified in one *Arabidopsis* accession is allocated a number (e.g., *RPP14*). Linked specificities in other accession lines that are directed to the same *P. parasitica* isolate are distinguished by a different decimal digit. This simplifies reference to an increasing number of *RPP* specificities being identified but makes no assumptions about whether they are allelic variants of a single gene or different genes in a complex locus.

The mapping of the resistance in accession Oy-0 to *Noco2* is of low resolution since it is based on a population of only 82 Col-0 × Oy-0 *Noco2*-susceptible F_2 gametes (total population size of approximately 650 F_2 plants). However, linkage of resistance to the regions containing *RPP4/RPP5* and *RPP1/RPP10/RPP14* is clear and is in agreement with the segregation data derived from two different populations, the Oy-0 × Nd-0 RIs and the Col-0 × Oy-0 F_2s. The RIs have also been tested for their reaction to the *Cala2* isolate of *P. parasitica* (Thomas Debener, unpublished results). The segregation profiles of resistant and susceptible lines were identical to that of *Noco2* except in two RI lines. These data suggest that the *Cala2*-recognition genes in Oy-0 are identical or closely linked to *RPP14.3* on chromosome 3 and *RPP5.2* on chromosome 4. Holub et al. (1994) also examined the segregation of the FN resistance phenotype of Oy-0 to *Cala2* in several F_2 populations. Their data were consistent with the presence of two independent *RPP* loci controlling resistance in Oy-0 to *Cala2*. The absence of recombination of the Oy-0 resistance with the locus *RPP2* in Col-0 (in 327 F_2 seedlings examined), indicated a strong linkage between at least one of the recognition specificities and *RPP2*, which mapped in Col-0 approximately 4 cM south of *AG* on chromosome 4 (Tör et al. 1994). Our data suggest that the Oy-0 locus on chromosome 4, which controls resistance to *Noco2* and which we have designated *RPP5.2*, is different in location and specificity from *RPP2*. However, it is likely that the second *Cala2*-recognition locus in Oy-0, which Holub et al. (1994) designated *RPP3*, is tightly linked or identical to a member of the *RPP1/10/14* cluster on chromosome 3. Further mapping analysis of F_3 families segregating for each *Noco2*-resistance locus will be needed to consolidate their positions in the genome and to avoid linkage distortion, which can be encountered when using only homozygous susceptible individuals. It will also allow us to assign a particular reaction phenotype to each locus individually and to the two loci in concert. Close examination of *Noco2*-susceptible seedlings of the inoculated Oy-0 × Nd-0 RI lines suggested the presence of a third very weak resistance segregating in this cross. This was not investigated further because the Oy-0 × Nd-0 combination exhibited a low level of polymorphism in the markers tested and was therefore not suitable for mapping studies. The "light and delayed" sporulation phenotype was not observed in the analysis of 256 Col-0 × Oy-0 F_2 seedlings (Table 3). It is possible, therefore, that the higher level of recombination that would be expected between linked loci in the F_8 RIs (Burr and Burr 1991) would reveal a linked weak resistance specificity that was not resolved in the F_2 analysis. Also, a third weak resistance locus might be easier to detect in the RIs, since 1/8 of lines would be essentially homozygous for only that gene (assuming it is unlinked to the other two *RPP* loci). However, the two populations were derived from different susceptible parents and this may strongly affect the expression of many components of the pathogen recognition including the *RPP* genes themselves.

The phenotypic classification scheme constructed by Holub et al. (1994) and utilized in the present study allows characterization of interaction phenotypes to a finer definition than a simple binary classification of resistance and susceptibility and demonstrates that there is gradation from full compatibility to incompatibility. In this study, resistance ranged from the strong FN phenotype exhibited by Oy-0 to the FDL phenotype of Bch-1 in response to *Noco2* inoculation. In Oy-0 the light flecking necrosis was correlated with very efficient containment of *Noco2* mycelium, whereas in Bch-1 extensive mycelial development was tolerated by the plant. It is probable that the strong pitting (PN) phenotype of Ws-0 and Pr-0 that was visible earlier and more distinctly than the FN phenotype of Oy-0 is the result of more host cells reacting in the formation of the lesion, as was shown in the microscopic analysis of inoculated cotyledons. It will be of interest in future studies to examine the different factors involved in determining the strength of the host response. For example, the more pronounced chlorosis of Pr-0 cotyledons, compared with that of Ws-0, in response to *Noco2* inoculation may be the result of intrinsic differences in the interaction of *RPP14.1* and *RPP14.2* with specific plant signals, although it is also likely that the different genetic backgrounds will affect the phenotypic expression of these genes.

The genetic data from several closely studied interactions controlled by single *RPP* loci (Parker et al. 1993b; Holub et al. 1994) show that partial (or semi-) dominance is a common feature of *RPP* gene–mediated resistance, and is probably the case for the monogenic resistances of Ws-0, Pr-0, Po-1, and Ge-1 described in this study. So, in these interactions, *RPP* gene dosage has a key rate-limiting effect on the expression of resistance. The variation observed in the macroscopic phenotypes between individual F_1 seedlings of a particular cross (Table 2) may be due to the sharp dose-dependence of the resistance phenotype on the respective *RPP* gene. Detailed microscopic analysis of individual parental and F_1 seedlings will determine whether the extent of mycelial development in all F_1 seedlings is intermediate between the resistant and susceptible parents, even though in some seedlings it does not lead to the production of sporophores. Of course, the final response of the plant will also depend on other factors such as the level of expression of the *Avr* determinants and the efficiency of delivery of fungal *Avr*, or indeed compatibility signals, to the plant. There may also be many different ways in which the *RPP* gene product can interact with other signaling components and downstream defense responses.

Our future aim is to clone the *RPP* genes involved in recognition of *Noco2* so we can critically address questions about their expression and function at the molecular level, as well as examine interactions of *RPP* genes with other components, and non-allelic interactions between defined *RPP* genes. This will lead to a greater understanding of the mechanisms controlling disease resistance.

MATERIALS AND METHODS

Plant material and cultivation, maintenance of *Peronospora parasitica Noco2,* and pathogenicity tests.

The origins of the Columbia (Col-0), Landsberg-*erecta* (La-*er*), Wassilewskija (Ws-0), Pr-0, Oy-0, Nd-0, and Ji-1 acces-

sions were as reported by Parker et al. (1993b). All other accessions used in this study were obtained from the Nottingham *Arabidopsis* Stock Center (Nottingham, UK). F_8 recombinant inbred lines (RIs) made between Nd-0 and Oy-0 were a kind gift from Jeff Dangl (Max-Delbrück Laboratory, Cologne, Germany). Segregation data from the Oy-0 × Nd-0 RIs inoculated with *P. parasitica* isolate *Cala2* were given by Thomas Debener (Federal Centre of Breeding Research on Cultivated Plants, Ahrensburg, Germany). An F_2 population made between Col-*gl* and Pr-0 was obtained from Shauna Sommerville (Carnegie Institute, Stanford, CA). All other F_2 populations described in this paper were generated for this study. The conditions for plant cultivation, maintenance of *Peronospora parasitica* isolate *Noco2*, and the pathogenicity tests were essentially as described previously (Parker et al. 1993b). Individual parental, F_1, and F_2 seedlings were drop-inoculated by means of the cotyledon assay (Dangl et al. 1992; Parker et al. 1993b). F_3 families (20 to 25 seedlings) were inoculated by spraying to run off a suspension of 5×10^4 *Noco2* conidia per ml in distilled water. Susceptible plants were rescued from *Noco2* infection by removing heavily infected organs, treating the plant with a drop of fungicide (0.1 mg/ml a.i. formulated as Ridomil, Ciba-Geigy Corp., Research Triangle Park, NC), and replacing to normal plant cultivation conditions. Reaction phenotypes of *Noco2*-inoculated cotyledons were scored according the classification of Holub et al. (1994). They presented five major classes of resistant reaction: necrotic pits (PN), which were visible as early as 3 days after inoculation; necrotic cavities (CN), a more diffuse appearance of necrotic flecks (FN); necrotic flecks accompanied by delayed and sparse fungal sporulation (FDL); and necrotic flecks with rare and extremely sparse fungal sporulation (FR). The susceptible phenotype was classed as the appearance of early (after 3 days) and ultimately (by 7 days) heavy sporulation (EH).

Light microscopy.

Development of the fungus was observed in whole infected cotyledons or seedlings stained with lactophenol-trypan blue and destained with chloral hydrate as described previously (Koch and Slusarenko 1990). Material was mounted in chloral hydrate and examined by means of phase-contrast optics on a Zeiss Axioskop microscope.

Isolation of plant genomic DNA.

Plant genomic DNA was isolated from glasshouse-grown plants using a rapid "flower head preparation" method. Inflorescences were ground through a sap extractor in a standard proteinase K buffer (Martienssen et al. 1989) into a 1.5-ml Eppendorf microcentrifuge tube and shaken at 28°C for 30 min. Samples were then extracted for 10 min at 37°C with phenol-chloroform buffered with Tris-EDTA (TE) containing 0.1 M NaCl. The aqueous phase was precipitated with isopropanol, the pellet resuspended in TE and then precipitated with ethanol. The final precipitate was resuspended in TE buffer. Genomic DNA prepared in this way was suitable for restriction enzyme digestion and polymerase chain reaction (PCR) analysis.

Cosmid and λ RFLP markers.

Cosmid clones (Hauge and Goodman 1992; Nam et al. 1989) and λ markers (Chang et al. 1988) were obtained from Renate Schmidt (Cambridge Laboratory, Norwich, UK). The restriction fragment length polymorphism (RFLP) marker pAT3-89.1 (Shirley et al. 1992) was obtained from Brenda Shirley (Virginia Polytechnic Institute and State University, Blacksberg, VA). Markers mi358 and mi413 in pUC119 were a kind gift from Robert Whittier (Mitsui Plant Biotechnology Research Institute, Tsukuba, Japan). Cosmid and plasmid DNA was extracted using the alkaline lysis method (Maniatis et al. 1982) and λ clone DNA was extracted according to Maniatis et al. (1982). The "Expressed Sequence Tag" (EST) marker, ve021, was generated from Col-0 DNA template with primer information kindly given by David Bouchez (INRA, Versailles, France). The amplification product (250 bp) was used as a probe. Total radiolabeled DNA probes were prepared by random hexamer primed labeling (Pharmacia, St. Albans, UK) and used to probe plant genomic DNA blots. For nucleic acid hybridizations, plant genomic DNA from parental plants and individuals from the various mapping populations was digested with appropriate restriction enzymes. Restriction digests, Southern blotting, and hybridizations were carried out by means of standard techniques (Ausubel et al. 1990).

PCR-based markers.

Ten nanograms of DNA isolated from parental plants, from F_2 individuals, or from F_3 families, was used as template for PCR amplification with oligonucleotide primers designed by Konieczny and Ausubel (1993) to generate "co-dominant cleaved amplified polymorphic sequence" (CAPS) markers, and by Bell and Ecker (1994) to amplify microsatellite sequences. The amplification reactions were carried out in a 20-µl volume containing 10 mM Tris/HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 100 µM each dNTP, 0.2 µM primer, and 1 unit *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT). The amplification conditions were 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C for 40 cycles in a Perkin-Elmer Cetus thermocycler. The 40 cycles were followed by 10 min at 72°C and the reactions were stored at 4°C. The CAPS primer-derived PCR products were digested with the appropriate restriction enzyme and analyzed on a 1.5% agarose gel. The microsatellite primer-derived PCR products were analyzed on a 4% agarose gel. Amplification conditions for a 600-bp product with the oligonucleotide primer OPA2 (Operon Technologies, Alameda, CA) that had been mapped previously by Tör et al. (1994) were optimized with Nd-0 (the original parent), Ws-0, and Col-0 DNA. A 600-bp product was reliably amplified in Nd-0 and Ws-0 DNA but not in Col-0 DNA, by means of 0.4 µM final primer concentration in a 20-µl reaction and the amplification conditions described above.

Mapping analysis.

The percent recombination between markers in selected F_2 plants, corresponding F_3 families, or RI plants was examined to identify linkage groups. Individual cross-over points were also scrutinized for anomalous recombination events. The data were analyzed with the MapMaker program (Lander et al. 1987) version 2.0, using a two-point analysis with a log-likelihood threshold of 3.0 and the Kosambi mapping conversion function of genetic recombination frequencies to cM values. The marker genotypes of the *Noco2*-resistant individuals in each mapping population were entered as unknowns in the F_2 intercross data.

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