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Phenotypic characterization and molecular mapping of the *Arabidopsis thaliana* locus *RPP5*, determining disease resistance to *Peronospora parasitica*

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Summary

***Peronospora parasitica* causes downy mildew on crucifers. An isolate of *P. parasitica* (denoted NoCO2) was identified that infected *Arabidopsis* plants of the land race Columbia (Col-0) but not plants of land race Landsberg *erecta* (La-*er*). Segregation analysis of F_2 plants derived from a La-*er* × Col-0 cross established that the resistance was inherited as a single locus, denoted *RPP5*. Macroscopic and microscopic examinations of inoculated La-*er* and Col-0 cotyledons showed that restriction of fungal growth in La-*er* was accompanied by massive callose accumulation and death of plant cells in direct contact with points of attempted fungal penetration. La-*er* × Col-0 F_1 plants exhibited an intermediate resistance response in all aspects of fungal development, indicating that *RPP5* is semi-dominant in its action. F_8 recombinant inbred lines generated between La-*er* and Col-0 were used to map *RPP5* to a narrow interval (<1.1 cM) on chromosome 4, utilizing existing restriction fragment length polymorphic (RFLP) markers and newly generated random amplified polymorphic DNA (RAPD) markers. The data provide a basis for the isolation of the *RPP5* locus by positional cloning as a first step towards understanding recognitional specificity in plant–pathogen interactions at a molecular level.**

Introduction

Genes for resistance to plant pathogens have long been recognized (Biffen, 1905) and selection for disease resistance genes plays a crucial role in crop improvement. However, little is understood about their mode of action at the molecular level.

Recent results from several laboratories have demonstrated that *Arabidopsis* is a host for all the major plant pathogens including viruses (Li and Simon, 1990; Sosnova and Polak, 1975), bacteria (Davis *et al.*, 1991; Parker *et al.*, 1993a; Simpson and Johnson, 1990; Tsuji *et al.*, 1991), fungi (Dangl *et al.*, 1992; Koch and Slusarenko, 1990a, 1990b) and nematodes (Sijmons *et al.*, 1991). Further studies have shown that there is natural variation for resistance and susceptibility among *Arabidopsis* land races for the first three groups of pathogens (Crute *et al.*, 1992; Debener *et al.*, 1991; Dong *et al.*, 1991; Koch and Slusarenko, 1990a; Parker *et al.*, 1993b; Simon *et al.*, 1993; Whalen *et al.*, 1991) and in some cases resistance has been shown to be controlled by a single genetic locus (Crute *et al.*, 1992; Debener *et al.*, 1991; Tsuji *et al.*, 1991). The development of *Arabidopsis thaliana* as a model plant to study the basic mechanisms of host–pathogen recognition now allows the use of molecular genetic strategies to dissect previously intractable aspects of plant disease processes.

Peronospora parasitica, a member of the oomycete family *Peronosporaceae*, is an obligate biotroph and causes downy mildew in *Cruciferae* (Channon, 1981). It has recently been shown to infect and complete its life cycle in *Arabidopsis* with the production of asexual and sexual spores (Koch and Slusarenko, 1990a). Additional studies by Crute *et al.* (1992) have shown that there are at least six distinct isolates of *P. parasitica* which are able to cause disease on different *Arabidopsis* land races. Based on the genetic analyses of these interactions it has been hypothesized that at least eight different genetic loci in the host can specify resistance against the different isolates of the fungus. These loci have been termed *RPP1-8* (Crute *et al.*, 1992). The genetic diversity exhibited by both the plant and pathogen, coupled with the ability to clone genetic loci that encode unknown proteins by employing the techniques of map-based cloning (Arondel *et al.*, 1992; Giraudat *et al.*, 1992), genomic subtraction (Sun *et al.*, 1992) and T-DNA tagging (Feldmann *et al.*, 1989; Koncz *et al.*, 1990; Yanofsky *et al.*, 1990) in *Arabidopsis*, provides an unparalleled opportunity to isolate genes that specify disease resistance.

Our research has focused on the NoCO2 isolate of *P. parasitica* which was originally isolated in Norwich from naturally infected plants of the Columbia (Col-0) land race. In this paper we report the phenotypic and microscopic characterization of interactions between this fungal isolate and both the susceptible Columbia (Col-0)

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and resistant Landsberg *erecta* (La-*er*) land races. In addition, we demonstrate that resistance to *P. parasitica* NoCO2 is controlled by a single semi-dominant locus that has been designated *RPP5.* Finally, as a prelude to map-based cloning, we demonstrate the utility of recombinant inbred lines of *Arabidopsis* to identify molecular markers that are very closely linked (<1.0 cM) to the *RPP5* locus.

Results

Phenotypic characterization of P. parasitica *NoCO2 on Col-0 and La-*er *plants*

A natural downy mildew infection caused by *P. parasitica* was discovered on *Arabidopsis* Col-0 plants growing in the glasshouse in Norwich. Figure 1 shows the characteristic downy appearance of sporangiophores bearing asexual spores (conidia) on the leaves and cotyledons. Viable conidia were recovered from infected material which had been frozen at –20°C and the resulting *P. parasitica* isolate (denoted NoCO2, see Experimental procedures) was obtained and bulked from a single infected Col-0 cotyledon to minimize the risk of culturing a genetically heterogeneous population.

The spectrum of *Arabidopsis* responses to NoCO2 was tested by inoculating cotyledons of different *Arabidopsis* land races with a suspension of freshly harvested conidia under controlled environmental conditions. Land races Col-0, Niedersenz (Nd-0), RLD, Kas-1, Li-6 and Weiningen supported profuse asexual sporulation after 4–6 days whereas land races La-*er*, Wassilewskija (Ws-0), Ji-1, Oy-0, Pr-0 and Mt-0 were resistant to NoCO2 infection. The land races Col-0 (susceptible to NoCO2) and La-*er* (resistant to NoCO2) are well characterized with respect

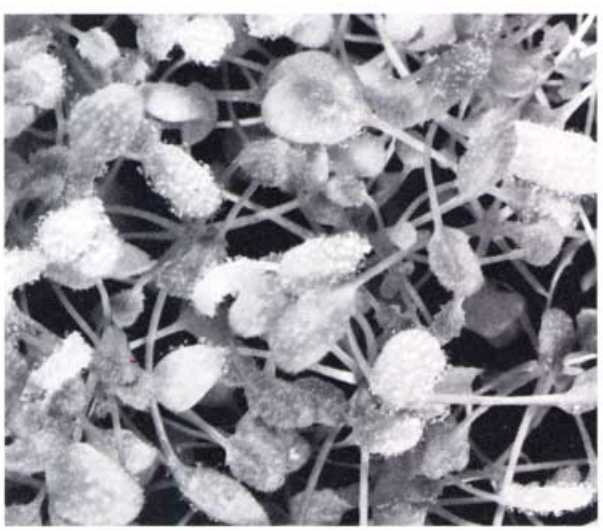


Figure 1. Downy mildew on Col-0 plants caused by *P. parasitica* NoCO2.

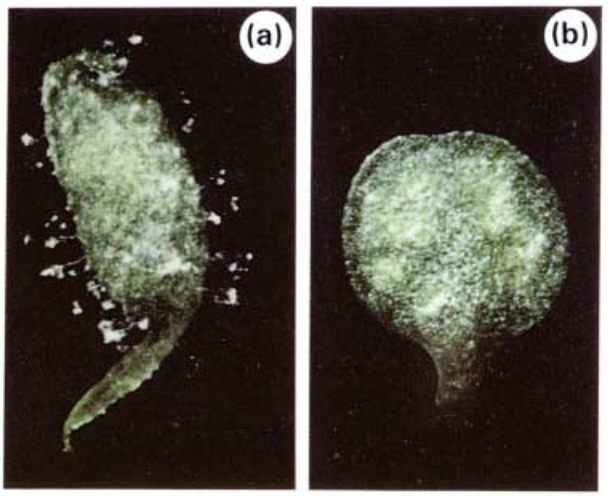


Figure 2. Reaction phenotypes of a Col-0 cotyledon (a), showing profuse asexual sporulation in the absence of plant cell necrosis, and a La-*er* cotyledon (b), showing diffuse necrosis in the absence of asexual sporulation, 6 days after inoculation with *P. parasitica* NoCO2.

to visible (Koornneef, 1990) and restriction fragment length polymorphism (RFLP) (Chang *et al.*, 1988; Nam *et al.*, 1989) genetic markers and were therefore used for a more detailed segregation analysis of the reaction phenotypes.

Cotyledons of Col-0 seedlings supported profuse asexual sporulation 4–6 days after inoculation with NoCO2 as shown in Figure 2(a). At this time point there was no visible necrosis of the infected tissue. However, from 7 days onwards infected cotyledons became necrotic and eventually collapsed. If seedlings were maintained in conditions of high humidity and low temperature, sporulation on uninoculated leaves was observed after 5–9 days indicating a rapid systemic spread of the fungal infection. Col-0 leaves were readily infected with NoCO2 conidial suspensions but early experiments showed that the intensity and timing of asexual sporulation were less consistent than on the cotyledons. In contrast to Col-0, La-*er* cotyledons did not support sporulation up to 9 days after inoculation and a diffuse necrosis of cotyledon cells was observed after 4 days which became more distinct after 6 days (Figure 2b). The compatible and incompatible interactions were therefore clearly distinguished, enabling us to score for resistance in segregating progeny.

*Inheritance of resistance to NoCO2 in crosses of Col-0 and La-*er *plants*

The F_1 progeny, and a segregating F_2 population derived from a cross between La-*er* and Col-0 plants, were inoculated with NoCO2 and scored for their reaction phenotypes at daily intervals. The results in Table 1 show that F_1 plants exhibited an intermediate response to NoCO2 infection. Plants failed to support early (4 days) and pro-

Table 1. Phenotypic characterization of Col-0, La-*er* and F_1 and F_2 progeny from a La-*er* × Col-0 cross, after inoculation with *P. parasitica* NoCO2

Plant type	Phenotype	Plants tested	Symptom expression
Col-0	Sensitive	10	10 No necrosis, profuse sporulation (>15 sporophores per cotyledon)
La-*er*	Resistant	12	12 Necrosis, no sporulation
F_1	Intermediate	25	3 Weak necrosis, no sporulation 22 Weak necrosis, sparse sporulation (<5 sporophores per cotyledon)
F_2	Segregating	308	76 Necrosis, no sporulation 62 Weak or no necrosis, no sporulation 98 Weak necrosis, sparse sporulation 72 No necrosis, profuse sporulation

Cotyledons were inoculated with a conidial suspension at a concentration of 5×10^4 ml^{-1} and symptom expression recorded 6 days after inoculation.

fuse asexual sporulation characteristic of fully sensitive Col-0 plants, but necrosis was less distinct at 4 and 6 days than in the resistant La-*er* plants. Also, late (visible at 6 days), very sparse, sporulation was observed on the cotyledons of 22 out of 25 F_1 plants tested.

Segregation analysis of F_2 plants showed that one quarter (72/308) were fully susceptible to NoCO2, exhibiting the phenotype of the sensitive parent, Col-0 (Table 1). The remainder were fully or partially resistant to NoCO2 infection. An intermediate response between La-*er* and Col-0 was observed in approximately one half (160/308) of the plants tested, measured by a reduction in the severity of necrosis and by late and sparse sporulation in a proportion (98/160) of these plants.

A 3:1 segregation ratio of resistance to sensitivity in F_2 plants (Table 2) is consistent with the interpretation that a single locus controls resistance in La-*er* to NoCO2. The resistance locus has been denoted *RPP5* in accordance with the interactive model proposed by Crute *et al.* (1992) for the interaction between *Arabidopsis* and *P. parasitica*. Also, the phenotype of the F_1 plants and the F_2 segregation data show that resistance is semi-dominant. However, a macroscopic assessment of F_1 and F_2 plant reactions suggests that plants heterozygous for *RPP5* exhibit a degree of phenotypic variation which makes it difficult to distinguish all of them unequivocally from plants homozygous for *RPP5*. Progeny testing of F_3 families would be necessary to assign accurately a genotype to these plants.

Table 2. Segregation analysis of *RPP5* in F_2 progeny and recombinant inbred lines (RIs) of a La-*er* × Col-0 cross

		Observed	Expected (3:1)	χ^2
F_2	Resistant	236	231	0.43 (ns)
	Sensitive	72	77	
		Observed	Expected (1:1)	χ^2
RIs	Resistant	129	144	3.12 (ns)
	Sensitive	159	144	

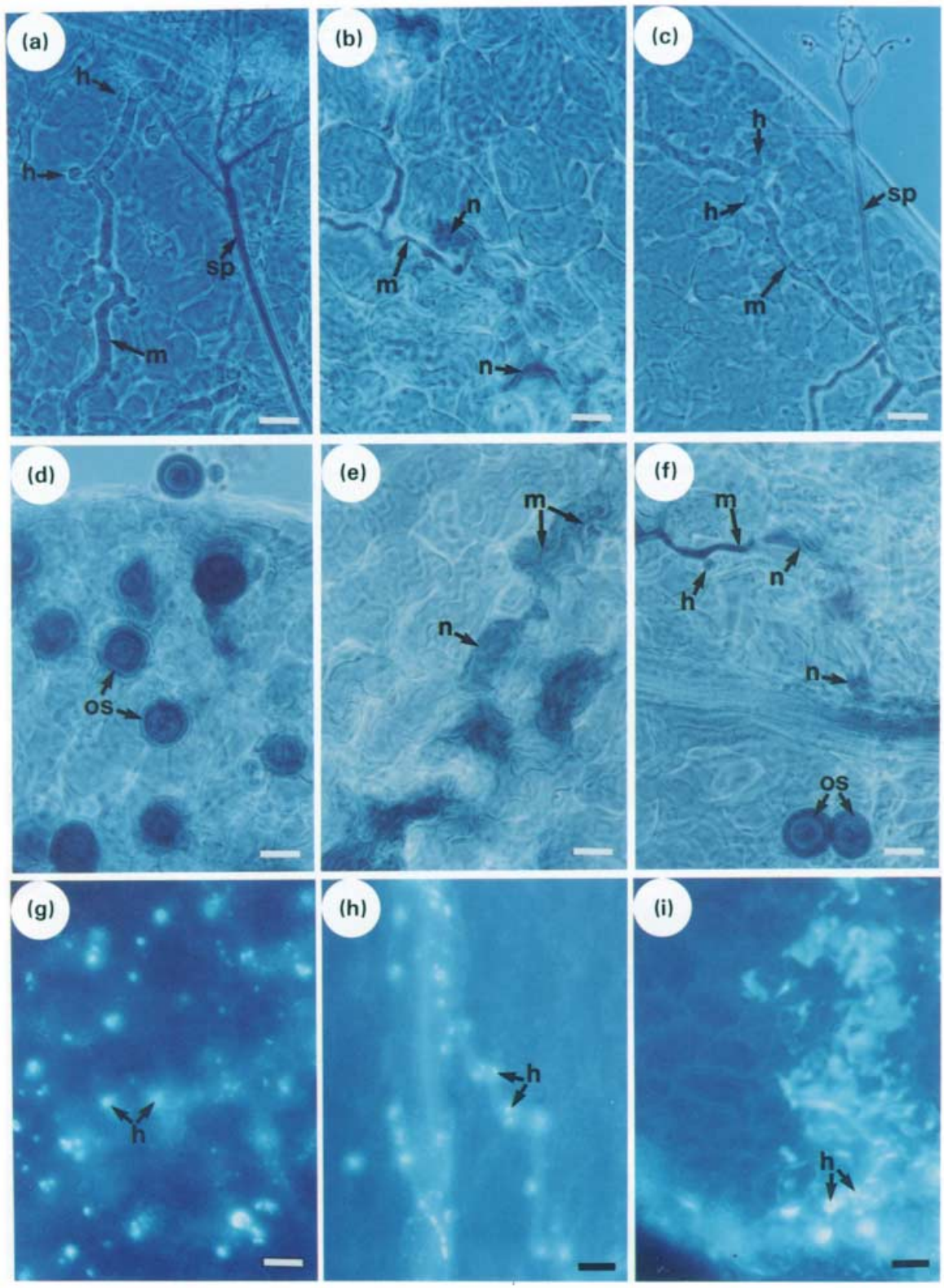
ns = not significant at $P = 0.05$.

Microscopic characterization of sensitive, resistant and intermediate plant phenotypes

The extent of fungal development in the compatible and incompatible interactions and in plants with an intermediate phenotype was examined microscopically.

Whole cotyledons of Col-0, La-*er* and La-*er* × Col-0 F_1 plants which had been inoculated with NoCO2 were stained with lactophenol-trypan blue at 4 and 7 days after inoculation and viewed under the microscope. The trypan blue stain is particularly informative because it not only stains fungal mycelium but is also selectively retained by necrotic plant cells and cells which have suffered membrane damage (Keogh *et al.*, 1980).

Representative results of microscopic examinations are shown in Figure 3. Four days after inoculation, cotyledons of Col-0 plants supported extensive fungal growth (Figure 3a). An intercellular mycelium with haustoria protruding into plant cells had ramified through the mesophyll and epidermal cell layers (Figure 3a). Fully developed sporangiophores (between 5 and 15 per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cotyledon) were also observed (Figure 3a) and were most abundant at the edges of cotyledons. By 7 days the fungal mycelium had grown through every part of the cotyledon (Figure 3d). In most but not all cotyledons, hyphae which had differentiated into the sexual reproductive structures, oogonia and paragynous antheridia (Koch and Slusarenko, 1990a), as well as fully formed oospores, were observed (Figure 3d). Plant cell necrosis or damage was not yet apparent.

In La-*er* cotyledons 4 days after inoculation, mycelium ingress was severely restricted within the mesophyll cell layer. The limited number of detectable hyphae were distorted (Figure 3b) and produced few haustoria which appeared to be encased, or were surrounded by necrotic plant cells (Figure 3b). By 7 days, some hyphae had progressed within the mesophyll to a limited extent, and to the epidermal layer, but these were nearly always accompanied by a trail of dead plant cells (Figure 3e). Occasionally hyphae appeared to grow beyond a cluster of dead cells towards the cotyledon border but sporangiophores were never observed.

Microscopic examination of cotyledons of La-*er* × Col-0 F_1 plants inoculated with NoCO2 showed clearly that fungal ingress was intermediate between the compatible (Col-0) and incompatible (La-*er*) interactions. At 4 days hyphae and haustoria were observed (Figure 3c) but development was less advanced than in Col-0 plants and was accompanied by limited plant cell necrosis. Occasional sporangiophores were detected on some cotyledons but again these were not as developed as in Col-0 plants (Figure 3c). By 7 days plant cell necrosis was more distinct although hyphae were still visible (Figure 3f). Between one and five sporangiophores were observed on cotyledons and occasional oospores were detected (Figure 3f).

The microscopic data confirm that phenotypic differences between the compatible and incompatible interactions observed at the macroscopic level are associated with differences in fungal ingress in the resistant and sensitive plant tissues and that expression of *RPP5* is semi-dominant in all aspects of NoCO2 development on the plant.

Callose accumulation in sensitive and resistant plants in response to NoCO2 infection

Callose is a 1,3-β-glucan of plant origin which has been found to accumulate as part of a plant's response to wounding or pathogen invasion (Aist, 1983; Kauss, 1987). The appearance of callose-containing papillae (cell wall depositions) has been implicated as a mechanism to prevent fungal penetration in several incompatible plant–fungal interactions (Bayles *et al.*, 1990; Hinch and Clarke, 1982; Lazarovits and Higgins, 1976; Skou *et al.*, 1984). It was therefore of interest to stain for callose in sensitive Col-0 and resistant La-*er* plants after inoculation with NoCO2.

Cotyledons of Col-0 and La-*er* seedlings were stained with decolorized aniline blue 3 days after inoculation with NoCO2 and viewed under the microscope for yellow fluorescence under illumination. In the compatible interaction between Col-0 plants and NoCO2, callose accumulation was limited to the area of direct haustorial contact with the plant cells as the mycelium progressed through the cotyledon (Figure 3g) and through the cotyledon stem (Figure 3h). It was not detected at the growing tips of the developing hyphae. In the incompatible interaction between La-*er* and NoCO2 callose deposition was much more intense than in Col-0 plants (Figure 3i). Fluorescence was strongest at the haustorium–plant cell interface but was also evident in the walls of mesophyll and epidermal cells in the area of attempted fungal growth. Plants inoculated with water did not show callose deposition over the course of the experiment (results not shown). The results indicate that the resistance in La-*er* controlled by *RPP5* is associated with an increased accumulation of callose in the areas of attempted fungal penetration.

Figure 3. Development of *P. parasitica* NoCO2 in cotyledons of Col-0 (sensitive), La-*er* (resistant) plants, and La-*er* × Col-0 F_1 (intermediate) plants. Cotyledons were stained with lactophenol-trypan blue 4 days (a–c) and 7 days (d–f) after fungal inoculation to show the infection process under phase contrast optics. Cotyledons were stained with aniline blue 3 days after inoculation (g–i) to show the presence of callose by bright fluorescence under UV illumination. Bar = 25 μm.

(a) Mycelial (m) ingress between Col-0 mesophyll cells showing protruding haustoria (h) and sporangiophore (sp) development.

(b) Attempted mycelial (m) ingress through the mesophyll of a La-*er* cotyledon. Note the distorted appearance of the hyphae and necrotic (n) mesophyll cells.

(c) Mycelial (m) development with haustoria (h) and immature sporangiophore (sp) in a La-*er* × Col-0 F_1 cotyledon.

(d) Oospore (os) development in Col-0.

(e) Characteristic trail of necrotic mesophyll cells (n) in the path of attempted mycelial (m) growth in La-*er*.

(f) Distorted mycelium (m) with haustoria (h) and occasional necrotic mesophyll cells (n), with sparse production of oospores (os) in La-*er* × Col-0 F_1 cotyledon.

(g) Callose accumulation at the haustorium–plant cell interface (h) in Col-0.

(h) Callose accumulation around haustoria (h) in the stem of a Col-0 cotyledon as the mycelium grows systemically.

(i) Massive callose accumulation in mesophyll and epidermal cells associated with attempted mycelial penetration and haustorium (h) formation in La-*er*.

Molecular mapping of RPP5

Recombinant inbred lines (RIs) between La-*er* and Col-0 which had been taken by single seed descent to the F_8 generation were available (Lister and Dean, 1993) and these were chosen to map *RPP5* as a first step towards map-based cloning of the locus.

Two hundred and ninety-two RIs were scored for their reaction phenotype to NoCO2. The segregation data are shown in Table 2. There was a 1:1 segregation of resistant to sensitive plants which is consistent with the presence of a single locus controlling resistance to NoCO2 and is in agreement with the F_2 segregation data (Table 2). Two hundred and eighty-eight RIs were either fully sensitive (profuse sporulation in the absence of plant cell necrosis) or fully resistant (plant cell necrosis in the absence of sporulation). The remaining four lines were of an intermediate phenotype or were a mixture of sensitive and resistant plants, perhaps indicating residual heterozygosity at the *RPP5* locus in these plants. These were not included in the segregation analysis.

One hundred RIs that had been scored for their response to NoCO2 were used to map *RPP5* relative to 63 selected RFLP markers from the Meyerowitz (Chang *et al.*, 1988) and Goodman (Nam *et al.*, 1989) collections which spanned chromosomes 1–5. Also, 200 RAPD markers were screened for polymorphisms between the La-*er* and Col-0 parental lines. Thirty of these (representing 48 loci) gave an easily scorable polymorphism and were tested further on 50 RIs for linkage to the RFLP markers and *RPP5*.

Linkage was detected with markers on chromosome 4 and *RPP5* mapped between RFLP markers m226 and g4539. One RAPD marker, $OPC18_{540}$, exhibited complete linkage with *RPP5*. A linkage map of chromosome 4 based on these data is shown in Figure 4(a). The remaining RIs were then tested for recombinants on each side of the *RPP5* locus. Five recombinants between *RPP5* and m226 were identified of 289 RIs tested and one line of 152 RIs showed recombination between *RPP5* and g4539. This placed *RPP5* 0.9 cM 'below' m226 as shown in Figure 4(b). We then selected for recombinants between *RPP5* and the *AGAMOUS* (*AG*) locus utilizing the 'CAPS' ('co-dominant cleaved amplified polymorphic sequence') strategy devised by Konieczny and Ausubel (1993) to score for polymorphisms at the *AG* locus. In this procedure, oligonucleotide primers have been designed to amplify a fragment of a single mapped DNA sequence in both Col-0 and La-*er* lines. The PCR product is then cleaved by a restriction enzyme in a land race-specific manner, producing a co-dominant marker. The *AG* gene lies approximately 6 cM below m226 (Hauge *et al.*, 1993). Primers specific for this gene (Konieczny and Ausubel, 1993) were used to amplify a

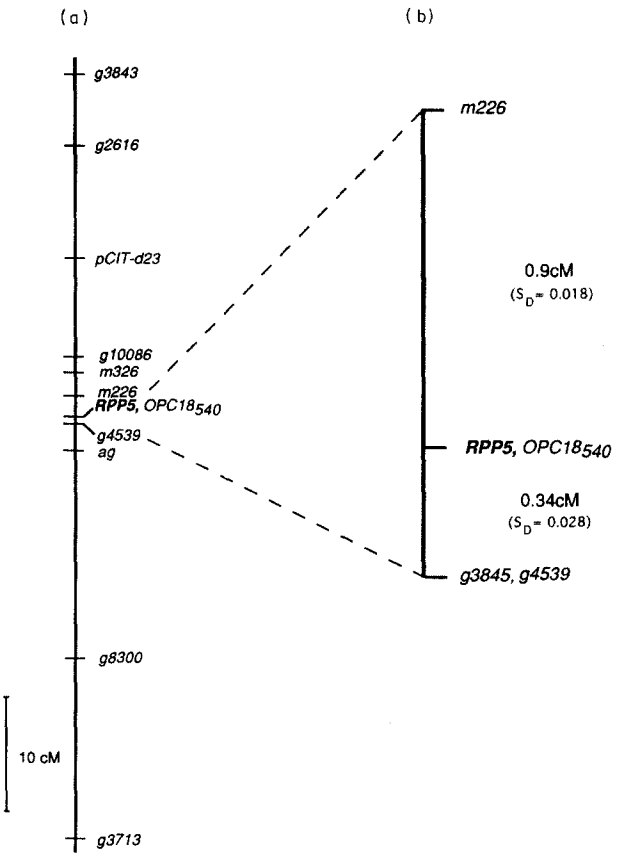

Figure 4. Linkage map of *Arabidopsis* chromosome 4 showing location of *RPP5*, relative to cosmid (g) and λ (m) RFLP markers and RAPD (OP) markers, based on the segregation analysis of La-*er* × Col-0 RIs.
(a) Mapping data derived from segregation analysis of RFLP markers on 100 RIs and RAPD markers on 50 RIs.
(b) Map position of *RPP5* relative to closely linked markers, from the analysis of 289 RIs (see 'Molecular mapping of *RPP5*' for details).

DNA fragment which was then cleaved by *Xba*I in La-*er* but not in Col-0 (results not shown). The RIs showing recombination between *AG* and *RPP5* were then probed with g4539 and g3845. An additional recombinant was found between g4539 and *RPP5* using this method. However, no recombinants were found between g3845 and g4539. This placed *RPP5* 0.34 cM 'above' g3845 and g4539. All seven lines carrying cross-overs closely linked to *RPP5* were then analysed with $OPC18_{540}$. No recombination was found between this marker and *RPP5*. These results define a relatively narrow genetic interval around *RPP5* (Figure 4b) as a basis for further positional analysis and molecular identification of the resistance locus.

Discussion

Classical genetic studies of numerous host–pathogen interactions have established that an incompatible response requires the expression of distinct 'resistance'

genes in the plant as well as 'avirulence' genes in the pathogen (Crute, 1985; Flor, 1971). However, the molecular basis of the recognition processes and consequent signalling mechanism which prevent the establishment of disease are poorly understood. A first step towards understanding these processes is to clone genetically defined resistance loci in order to analyse their role in plant–pathogen recognition.

We have identified a locus, *RPP5*, in *Arabidopsis* plants of the land race La-*er* which controls resistance to the NoCO2 isolate of the downy mildew pathogen *P. parasitica*. Segregation analysis of two independent populations, an F_2 population and F_8 recombinant inbred lines (Table 2), derived from crosses between La-*er* and the sensitive land race Col-0 have established the monogenic inheritance of *RPP5*.

Macroscopic and microscopic examinations of F_1 and F_2 plants showed that expression of *RPP5* is semi-dominant since the severity of the resistance response was clearly gene dosage-dependent. This suggests that *RPP5* encodes the key rate-limiting determinant of the resistance reaction and that any consequent signalling events are tightly regulated by *RPP5* activity and are not in themselves limiting the response. The mechanistic implications of semi- (incomplete) dominance have been discussed previously by Crute and Norwood (1986) in relation to genetically defined resistance genes in lettuce to downy mildew (*Bremia lactucae*). In the present study, saturation mutagenesis of La-*er* would help to assess how many other loci encoding proteins which operate downstream of *RPP5* are involved in maintaining resistance.

Microscopic analysis of compatible and incompatible plant responses was performed using lactophenol-trypan blue to stain infected cotyledons. Resistance in La-*er* was manifested as necrosis of plant cells, principally in the mesophyll layer, which were in direct contact with points of attempted fungal penetration. This contrasts with the resistance phenotype of land race RLD in response to inoculation with the 'WeLa' isolate of *P. parasitica* (Koch and Slusarenko, 1990a) in which individual epidermal cells became necrotic after initial fungal penetration. Indeed, one of the fascinating aspects of the *Arabidopsis –P. parasitica* interaction, first demonstrated by Crute *et al.* (1992) is the diverse range of reaction phenotypes with different plant–pathogen combinations. Several distinct resistance responses are now known to be due to the presence of different resistance loci (Crute *et al.*, 1992; Dangl *et al.*, 1992). It is predicted that these interact with a correspondingly large array of fungal avirulence genes, in accordance with Flor's original gene-for-gene hypothesis (Flor, 1955, 1971). It is of interest to note that the genetic incompatibility determined by *RPP5*, expressed as plant cell necrosis, is reminiscent of 'systemic acquired resistance' (SAR) of *Arabidopsis* plants to an otherwise compatible *P. parasitica* isolate following application of the synthetic immunizing compound 2,6-dichloroisonicotinic acid (Uknes *et al.*, 1992). Therefore, it appears that different inducing stimuli result in the same final expression of resistance. Future studies will determine whether the range in severity of *Arabidopsis* necrotic responses to *P. parasitica* controlled by distinct resistance loci, as well as the immunity of SAR, share signalling components or operate through different cellular pathways.

We show that resistance in La-*er* to NoCO2 is also associated with massive callose deposition compared with the compatible interaction of Col-0 plants, and therefore has features common with several previously studied crop plant reponses (Bayles *et al.*, 1990; Hinch and Clarke, 1982; Lazarovits and Higgins, 1976; Skou *et al.*, 1984) in which the early appearance of callose was coincident with a failure of fungal penetration. Callose, a 1,3-β-glucan polymer is formed by a plasma membrane localized enzyme, 1,3-β-glucan synthase, whose activity is stimulated by Ca^{2+} ions (Kauss, 1987). It is possible that recognition of NoCO2 by *RPP5* leads to an alteration in free Ca^{2+} levels and increased stimulation of callose synthesis at the cell surface. This would form an impermeable barrier to fungal penetraton. The limited accumulation of callose at the haustorial–plant cell interface in the compatible interaction suggests that this is not sufficient to prevent haustorial function. The experimental and genetic amenability of *Arabidopsis*, compared with more complex crop plant genomes, now enables us to test whether these plant cell responses are directly dependent on *RPP5* function or are a secondary and non-specific consequence of the initial resistance reaction. We are generating a Col-0 line into which a narrow interval of La-*er* DNA containing *RPP5* has been introgressed using a molecular marker-assisted strategy. It will then be possible to determine precisely the timing and sequence of cellular events in near-isogenic resistant and sensitive plants.

Recombinant inbred lines (RIs) were used to map *RPP5* as a prelude to chromosome walking to the locus because they offer several key advantages over F_2 plants (Burr *et al.*, 1988). They are essentially homozygous at each locus and therefore can be propagated indefinitely without further segregation of loci, whereas F_2 or backcross populations eventually become depleted. Also, linked loci are more likely to have recombined because multiple rounds of meiosis have occurred to attain homozygosity (Burr *et al.*, 1988; Haldane and Waddington, 1931). In addition, RIs allow the use of dominant polymorphic DNA markers such as RAPDs, as has been demonstrated in *Arabidopsis* by Reiter *et al.* (1992). In this study, the possible mis-scoring of heterozygotes of a

semi-dominant allele due to intrinsic variation in the intermediate resistant response (see Table 1) was avoided with RIs since the residual heterozygosity was low. Two hundred and eighty-eight out of 292 RIs were either fully sensitive or fully resistant at the F_9 generation. Of the remaining four lines, two RIs appeared to be uniformly heterozygous for *RPP5*. The other two lines were segretating for resistance and sensitivity and were assumed to have been heterozygous for *RPP5* in the F_8 generation. Therefore, we estimate approximately 1% residual heterozygosity at the *RPP5* locus at F_9. This is higher than the value of 0.2% estimated, based on the initial segregation analysis of 63 RFLP markers on 100 RIs. A more detailed analysis of the RIs will be published elsewhere (Lister and Dean, 1993).

RPP5 has been mapped on the La-*er* × Col-0 RIs to a narrow interval (<1.1 cM) on chromosome 4 using a combination of RFLP and RAPD markers. One RAPD probe, $OPC18_{540}$, was identified which co-segregated with *RPP5* in a total of 295 RIs examined, and therefore lies <0.19 cM from the resistance locus. This marker will serve as an anchor point for the fine molecular analysis of the genome in the area around *RPP5*. Additional recombinants close to *RPP5* are now being selected from F_2 populations using flanking genetic and molecular markers. The utilization of the PCR-based 'CAPS' strategy (Konieczny and Ausubel, 1993), as well as the visible markers *ara1* and *cs*, will greatly facilitate the identification of more recombinants within a defined genetic interval, and the definition of a physical interval within which *RPP5* must be located.

The extensive genetic diversity exhibited in the interaction between *Arabidopsis* and *P. parasitica* (Crute *et al.*, 1992) makes it an ideal model system to dissect the genetic determinants of plant–pathogen recognition. The phenotypic characterization and mapping of one genetically defined resistance locus, *RPP5*, described in this paper, is a first step towards understanding the molecular mechanisms involved in disease resistance.

Experimental procedures

Plant material and cultivation

Arabidopsis land race Columbia (Col-0) was originally obtained from Chris Somerville (Michigan State University, East Lansing, MI) and Landsberg *erecta* (La-*er*) from Maarten Koornneef (University of Wageningen, The Netherlands). Wassilewskija (Ws-0) was kindly given by Ken Feldmann (University of Arizona, Tucson, AZ), RLD and Weiningen by Alan Slusarenko (University of Zurich, Switzerland) and Pr-0 by Shauna Sommerville (Michigan State University, East Lansing, MI). Ji-1 is a local land race and Nd-0, Kas-1, Oy-0, Li-6, and Mt-0 were obtained from the *Arabidopsis* Information Service (Kranz and Kirchheim, 1987). Three hundred recombinant inbred lines (RIs) were generated by single seed descent to the F_8 generation from a segregating F_2 population derived from a cross between La-*er* and Col-0 plants (Lister and Dean, 1993). Seeds were bulked at the F_9 generation for this study. Seeds were sown on to damp Levington's compost and allowed to germinate under propagator lids without further watering until the seedlings had emerged. For pathogenicity tests seeds were sown directly into 2.5 cm × 3 cm compartments of seedling trays at a density of four to five seeds per compartment. Seedlings were grown in a growth chamber at 22°C (±1°C) and 65% relative humidity under an 8 h photoperiod with a light intensity of 180–250 μEinsteins m^{-2} sec^{-1}. Plants used for crosses were grown in the glasshouse at 23°C (±3°C) with supplementary lighting to give a 16 h day length.

Maintenance of Peronospora parasitica *NoCO2*

Col-0 seedlings growing in the glasshouse were found to be infected with *P. parasitica* in January 1991. Cotyledons and leaves bearing conidia were harvested and stored in microcentrifuge tubes at −20°C. Viable conidia were recovered from the frozen material by allowing it to thaw to room temperature and then adding 1 ml dH_2O and inverting the tube several times. The leaf material was removed and the spores (10^4 ml^{-1}) were inoculated on to 3-week-old Col-0 plants by placing drops of the suspension on to the cotyledons and leaves. Plants were incubated under a sealed propagator lid to achieve high relative humidity in a growth chamber at 16°C under an 8 h photoperiod with a light intensity of 150–200 μEinsteins m^{-2} sec^{-1}. Asexual sporulation was observed after 6 days and a single leaf bearing conidia was used to inoculate healthy seedlings and build up the inoculum.

Infected Col-0 material was given to Eric Holub and Ian Crute (Horticultural Research International, East Malling, Kent, UK) to include in their genetic analysis of the interactive phenotypes of different *Arabidopsis* land races with several *Peronospora peronospora* isolates (Crute *et al.*, 1992; Dangl *et al.*, 1992). The reaction phenotypes of the selected land races Col-0, Nd-0, La-*er*, Oy-0 and Ws-0 in response to NoCO have remained stable throughout the course of this study. However, the initial NoCO isolate (denoted NoCO1) appears to have differed from the present isolate as RLD plants were recorded as resistant in early infection experiments. The present *P. parasitica* isolate is therefore referred to as NoCO2.

NoCO2 was cultured at weekly intervals by harvesting 20–30 Col-0 seedlings showing sporulation in 50 ml plastic tubes. These were vortexed briefly in dH_2O and, after removal of plant material, conidia were pelleted by centrifugation at 1500 ***g*** for 5 min (Dangl *et al.*, 1992). The conidia were then resuspended to a concentration of 10^4 ml^{-1} and sprayed with an atomizer on to a dense lawn of 2–3-week-old Col-0 seedlings. These were incubated as described above and sporulation observed after 4–7 days.

Pathogenicity tests

Arabidopsis seedlings were inoculated with *P. parasitica* NoCO2 10–12 days after germination when the first two true leaves were emerging. Freshly harvested conidia were washed once in dH_2O as described above and made to 2×10^4 ml^{-1}. A drop (5 ml) of the conidial suspension was placed on each cotyledon and the seedlings incubated under sealed propagator lids for the duration of the experiment using the conditions described for

'Maintenance of *P. parasitica* NoCO2'. Plants were scored for asexual sporulation 3–9 days after inoculation.

Infected seedlings were recovered for seed production by removing cotyledons bearing sporangiophores and replanting the seedlings in fresh Levington's compost. Plants were then placed in a growth chamber or the glasshouse at 22–25°C with 16 h supplementary lighting in order to suppress fungal sporulation (Dangl *et al.*, 1992).

Light microscopy

Development of the fungus was observed in whole infected cotyledons stained with lactophenol-trypan blue and destained with chloral hydrate as described previously (Koch and Slusarenko, 1990a). Material was mounted in chloral hydrate and examined using phase-contast optics on a Zeiss 'Axioskop' microscope. In order to detect callose accumulation cotyledons were stained with decolorized aniline blue according to Lazarovits and Higgins (1976). Callose deposits were identified by their bright yellow fluorescence using a high-pressure mercury HBO 50 lamp (transmission range 430–500 nm).

Cosmid and λ RFLP markers

Cosmid clones were obtained from Brian Hauge (MGH, Boston, USA) (Hauge and Goodman, 1992; Nam *et al.*, 1989) and were cultured in the presence of 30 mg ml^{-1} kanamycin. The DNA was extracted using an alkaline lysis method (Maniatis *et al.*, 1982). λ clones were obtained from Elliot Meyerowitz (CIT, Pasadena, USA) and the DNA was extracted according to Maniatis *et al.* (1982). Total radiolabelled DNA was used to probe plant genomic DNA blots.

Isolation of plant genomic DNA

Plant genomic DNA was isolated from either glasshouse-grown plants or from sterile cultures grown in Gamborg's B5 medium with sucrose (Reiter *et al.*, 1992). Plant genomic DNA (3–5 g material) was prepared using the method of Tai and Tanksley (1991) except that the tissue was ground in liquid nitrogen and the RNase step was omitted.

Nucleic acid hybridization

Plant genomic DNA (2 μg) from both parents (La-*er* and Col-0) and from the RIs was digested with appropriate restriction enzymes. The digested DNA was separated on 0.8% agarose gels. Gel transfer to Hybond-N membranes (Amersham), hybridization and washing conditions were according to the manufacturer's instructions, except that the DNA was cross-linked to the filter by UV Stratalinker treatment (2400 μJ × 100; Stratagene) and baked at 80°C for at least 2 h. Radiolabelled DNA was prepared by random hexamer primed labelling (Pharmacia) according to the manfuacturer's instructions.

PCR-based markers

DNA from 50 RIs was used for templates for PCR amplification using single 10-mer oligonucleotide primers obtained from Operon Technologies (Alameda, CA). The reaction conditions were as described by Williams *et al.* (1990) for a 25 μl reaction volume containing 10 mM Tris/HCl pH 8.3, 50 mM KCl, 1.9 mM $MgCl_2$, 0.01% gelatin, 100 μM each dNTP, 0.2 μM primer and 1 unit Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). Amplification was performed using a Perkin Elmer Cetus thermocycler for 35 cycles. Each cycle consisted of 30 sec at 94°C, 30 sec at 31°C, 15 sec at 45°C and 1 min at 72°C. The 35 cycles were followed by 10 min at 72°C and the reactions were stored at 4°C until they were analysed on a 1.5% agarose gel.

AGAMOUS-specific oligonucleotide primers were constructed and used to amplify a 1366 bp fragment in La-*er* and Col-0 DNA according to Konieczny and Ausubel (1993). Subsequent treatment of the amplification product with *Xba*I cleaved La-*er* DNA into two fragments of 1073 bp and 293 bp, whereas Col-0 DNA remained intact (Konieczny and Ausubel, 1993).

Mapping analysis

The per cent recombination in the RI population was converted to a map distance using the Haldane and Waddington (1931) formula:

$$r = \frac{R}{2 - 2R}$$

where r is the map distance that would be calculated from a single meiosis and R is the fraction of recombinants. The conversion to centimorgans (cM) was then calculated using the Kosambi function (Koornneef and Stam, 1992). The data were analysed using the MAPMAKER program (Lander *et al.*, 1987) Version 2.0, choosing a log-likelihood threshold of 4.0.

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