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# Necrotic Lesion Resistance Induced by *Peronospora tabacina* on Leaves of *Nicotiana obtusifolia*

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## ABSTRACT

Heist, E. P., Zaitlin, D., Funnell, D. L., Nesmith, W. C., and Schardl, C. L. 2004. Necrotic lesion resistance induced by *Peronospora tabacina* on leaves of *Nicotiana obtusifolia*. *Phytopathology* 94:1178–1188.

Infection of *Nicotiana obtusifolia* plant introduction (PI) #555573 by the downy mildew pathogen *Peronospora tabacina* resulted in a compatible interaction, in which *P. tabacina* penetrated and freely colonized host leaf tissue. This interaction became incompatible 5 to 6 days later, with the appearance of necrotic lesions (NLs) and inhibition of pathogen growth and subsequent sporulation. NL development depended upon the presence of *P. tabacina* in host tissue, was not due to the effects of other microbes, and occurred co-incident in time with the pathogen's ability to produce asexual sporangia on a susceptible *N. obtusifolia* genotype. Inhibition of the necrotic response by CoCl<sub>2</sub> (a calcium channel blocker) and pathogen-induced transcription of a defense-related gene (*PR-1a*) suggested that necrosis was due to hypersensitive cell death in the host. In

contrast, *N. obtusifolia* PI#555543 did not exhibit hypersensitivity upon infection by *P. tabacina*, but rather developed characteristic symptoms of tobacco blue mold disease: chlorotic lesions accompanied by abundant pathogen sporulation. Disease reactions scored on PI#555573 × PI#555543 F<sub>2</sub> progeny inoculated with *P. tabacina* sporangia indicated that the resistance phenotype was due to the action of a single gene from *N. obtusifolia* PI#555573, which we have named *Rpt1*. To date, *Rpt1* is the only gene known to confer a hypersensitive response (HR) to *P. tabacina* infection in any species of *Nicotiana*. A survey of wild *N. obtusifolia* revealed that the HR to *P. tabacina* was expressed in the progeny of 7 of 21 (33%) plants collected in southern Arizona, but not in the progeny of plants originating from Death Valley National Park in California and the Big Bend National Park in west Texas.

*Additional keywords:* desert tobacco, oomycete, tissue specificity.

*Peronospora tabacina* D. B. Adam (syn. *P. hyoscyami* de Bary), an oomycete, causes an economically important downy mildew disease known as tobacco blue mold on cultivated tobacco (*Nicotiana tabacum* L.) and other species of *Nicotiana*. Like all members of the family *Peronosporaceae*, *P. tabacina* is an obligate biotroph that requires living host tissue to complete its life cycle.

Tobacco blue mold is a polycyclic disease and, during periods of weather favorable for disease development, *P. tabacina* can complete its life cycle in 10 days or less (30). Asexual sporangia, typically produced on the leaves and stems of infected plants, serve as both primary and secondary inoculum. On actively sporulating leaf tissue, *P. tabacina* can produce as many as one million sporangia/cm<sup>2</sup>, a major factor that can contribute to the complete devastation of infected tobacco crops (30). Although these sporangia are relatively short lived and can be killed by environmental factors such as ultraviolet light (30), they have been reported to travel long distances and cause disease several hundred kilometers from their site of origin (2,49). For these reasons, reducing or eliminating the production of asexual sporangia by *P. tabacina* is critical for effective disease control.

Disease resistance that is determined by single genes of major effect very often is associated with hypersensitive cell death in plants. Stakman (47) coined the term "hypersensitive response" (HR) to describe the observed reaction of wheat and other cereals to infection by *Puccinia graminis*. The HR involves rapid collapse

of host plant cells following recognition of pathogen-associated factors (elicitors) (6,13,14,50). Considerable evidence suggests that the HR is part of the plant's defense against disease, and requires active host metabolism (11,26,28). The HR can be considered a form of programmed cell death in plants, sharing similarities with apoptosis in animals (18,23), one of which is fragmentation of nuclear DNA late in the HR (33). Factors that can induce the HR following pathogen infection are highly sought for their potential use in plant disease-control strategies, and are especially important for controlling obligately biotrophic pathogens.

We report here that some genotypes of the desert tobacco, *N. obtusifolia* Martens & Galeotti, respond to foliar *P. tabacina* infections by developing necrotic lesions 5 to 6 days postinoculation (dpi), and that subsequent sporulation of the pathogen is drastically reduced or eliminated in these interactions. We present evidence that this resistance phenotype in *N. obtusifolia* plant introduction (PI) #555573 (hereafter designated PI73) results from the expression of the HR, and is due to the action of a single, partially dominant gene that we have named *Rpt1*.

## MATERIALS AND METHODS

**Plant material.** Seed of *N. obtusifolia* var. *obtusifolia* (= *N. trigonophylla* Dunal) (19) PI73 and *N. obtusifolia* var. *palmeri* (= *N. palmeri* Gray) (19) PI#555543 (hereafter designated PI43) were provided by V. Sisson of the Oxford Tobacco Research Station, Oxford, NC. *N. obtusifolia* accessions AusTRC303759 and AusTRC314549 were provided by P. Lawrence of the Queensland (Australia) Department of Primary Industries, and NIC439, NIC455, and NIC456 were provided by A. Graner, IPK-Gatersle-

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ben. Seed of all accessions was sown in 15-cm-diameter pots containing moistened Pro Mix BX (Premier Horticulture Inc., Red Hill, PA) and incubated in a plant growth chamber at a light intensity of 66  $\mu\text{E m}^{-2} \text{s}^{-1}$  at 25°C. Two to three weeks following seed germination, individual seedlings were transferred to 15-cm-diameter pots containing moist Pro Mix BX and maintained in growth chambers as previously described (24).

Mature seeds were collected from 21 individual plants of *N. obtusifolia* encountered in the wild in Arizona in January and May 2002. Seed from plants growing in Death Valley National Park (3 collections) and Big Bend National Park (12 collections) were provided by National Park Service (NPS) botanists Dana York and Joe Sirotnak, respectively, under NPS permit #BIBE-2002-SCI-0024. For each collection, plants grown in the greenhouse from wild-collected seed were observed to be morphologically uniform, and seeds were harvested and pooled from three to four plants to preserve existing genetic diversity. Seed stocks are maintained at the Kentucky Tobacco Research and Development Center, University of Kentucky.

**Maintenance of *P. tabacina* isolates.** The origins and characteristics of *P. tabacina* isolates used in this study are listed in Table 1. All isolates were maintained by two methods: (i) propagation on *N. tabacum* L. cv. KY14 in growth chambers (described below) and (ii) propagation on *N. repanda* in gnotobiotic (contaminant-free) associations as described previously (24). To maintain *P. tabacina* isolates by method (i), sporangia were collected by gently scraping the surface of sporulating lesions (on infected plants) with a wooden applicator stick, which then was swirled gently in a microcentrifuge tube containing sterile distilled water. Sporangial suspension (10 to 20  $\mu\text{l}$ ) was introduced into leaf panels of 8- to 14-week-old plants of *N. tabacum* cv. KY14 by infiltration using a hypodermic syringe equipped with a 20-gauge needle. Sporulation was induced 2 weeks postinoculation by misting the plants with sterile distilled water and placing them (or detached leaves) into premoistened plastic bags in the dark for 12 h. The resulting sporangia were used to inoculate new KY14 plants. This process was repeated biweekly to maintain isolates throughout the study.

**Plant inoculations.** Leaves of *N. obtusifolia* plants were inoculated with *P. tabacina* sporangia by either drop inoculation or infiltration. Water suspensions containing 4.5 to 5.5  $\times 10^4$  sporangia  $\text{ml}^{-1}$  were used for all inoculations and were obtained from either infected *N. tabacum* cv. KY14 plants in growth chambers or gnotobiotic associations, where indicated. Plants were inoculated with 4- $\mu\text{l}$  droplets essentially as described in Heist et al. (25).

For the pathogenesis-related (PR) gene induction experiments, plants were sprayed to runoff with *P. tabacina* sporangia in distilled water ( $\approx 5 \times 10^4 \text{ ml}^{-1}$ ) and then maintained as described above. Beginning at 1 dpi, inoculated, asymptomatic leaves from the innermost two whorls were harvested from individual plants every 24 h, frozen on solid  $\text{CO}_2$ , and stored at  $-80^\circ\text{C}$  prior to RNA extraction.

**Geographical survey of *N. obtusifolia* response to *P. tabacina* infection.** Forty-three accessions of desert tobacco, representing wild collections from Arizona, Texas, and California, as well as accessions from the U.S. Department of Agriculture (USDA), AusPGRIS, and IPK-Gatersleben (Table 2), were grown in the greenhouse until 6 weeks of age. All plants then were transferred to a walk-in growth chamber designed for blue mold containment for an additional 5 days prior to inoculation with *P. tabacina* sporangia. Light intensity in the chamber was 80  $\mu\text{E m}^{-2}$  (LI-250 light meter; Li-Cor Inc., Lincoln, NE), with a 12-h photoperiod and a temperature of 21 to 23°C. Inoculum was prepared from an infected leaf of *N. tabacum* cv. KY14. Three leaves on each of five plants were inoculated at four sites per leaf with 4  $\mu\text{l}$  of a water suspension of sporangia ( $5 \times 10^4 \text{ ml}^{-1}$ ). Inoculated plants (and mock-inoculated controls) were lightly misted with water

and sealed in large opaque plastic tubs for 15 h, after which they were returned to the lighted shelves. All plants were rated for disease symptoms at 8 dpi, and the inoculated leaves were removed from each plant and treated as described above to assess the degree of pathogen sporulation. Sporulation was rated the next day on a four-point scale, where 1 = no visible sporulation, 2 = small patches of sporangiophores present around the lesion margin, 3 = zone of sporulation extending up to 1 cm outward from the lesion border, and 4 = heavy sporulation encompassing the entire symptomatic area or leaf surface.

**Assessment of responses.** Ten 40-day-old plants of *N. obtusifolia* accessions PI43 and PI73 were inoculated with each of the six *P. tabacina* isolates (Table 1) by either drop or infiltration (five plants for each method per isolate). An additional 10 plants of each accession were inoculated with sporangia that had been killed by holding them at 4°C for 1 week. Plants of *N. tabacum* cv. KY14 also were inoculated to confirm that the sporangia had been inactivated. Plant responses and disease symptoms were recorded daily for 1 month. At 8 dpi, half of the plants from each treatment were placed in conditions conducive to sporulation for *P. tabacina*.

To test whether plant responses were affected by microbial contaminants in inoculum taken from nonaxenic sources (e.g., infected plants raised in growth chambers), 10 40-day-old plants each of accessions PI43 and PI73 were infiltrated with sporangia of *P. tabacina* isolate KY79 collected from gnotobiotic associations (24). An additional 10 plants of each *N. obtusifolia* accession served as controls and were inoculated with cold-killed sporangia of isolate KY79 collected from gnotobiotic associations.

To determine age dependence of *N. obtusifolia* plant responses to *P. tabacina* infections, 70 plants each of accessions PI43 and PI73 were grown and, at weekly intervals for 7 weeks, subsets of 10 plants were drop inoculated with *P. tabacina* KY79. Characteristics of the lesions were observed for 2 weeks following inoculation. At 8 dpi, half of the infected plants were placed into conditions conducive to pathogen sporulation and the results recorded.

**Timing of necrotic response in relation to pathogen sporulation.** Seventy 45-day-old plants each of accessions PI43 and PI73 were drop inoculated with sporangia of *P. tabacina* KY79. Each day for 7 days, beginning at 1 dpi, a subset of 10 plants from each accession was placed into conditions conducive to *P. tabacina* sporulation as described above.

**Effects of metabolic inhibitors on the necrotic response.** At 4 dpi, 10 inoculation sites were treated with each of the following metabolic inhibitors: 0.5 mM cobalt chloride ( $\text{CoCl}_2$ , a calcium channel blocker) (3), 5  $\mu\text{M}$  cycloheximide (an inhibitor of 80S ribosomes), and 50  $\mu\text{M}$  sodium orthovanadate (an ATPase/phosphatase inhibitor) (22,26). The inhibitors were infiltrated within 1 cm of the site where the pathogen initially was introduced by infiltration (easily discerned by the needle mark), to the point where a 1-cm water soak mark was visible. Treatments with inhibitors or water were made on the same leaf (four inoculation sites per leaf, each treated with a different inhibitor or water). Effects of inhibitors were noted each day following treatment for

TABLE 1. Six isolates of *Peronospora tabacina* examined in this study

Isolate	Year collected	Origin <sup>y</sup>	Reaction to metalaxyl <sup>z</sup>
KY79	1979	Georgetown, KY	+
KY93	1993	Composite	-
Logan95	1995	Logan County, KY	-
CONN96	1996	Connecticut	-
Shelby97	1997	Shelby County, KY	-
Texas98	1998	Texas	+

<sup>y</sup> Location where isolates were collected. Composite = a mixture of metalaxyl resistant isolates that were collected from various locations in Kentucky during the 1993 epidemic.

<sup>z</sup> Isolates sensitive (+) or resistant (-) to the fungicide metalaxyl.

3 days (until 7 dpi). The plants then were placed in conditions conducive to pathogen sporulation. Additional controls consisted of uninoculated plants that were treated similarly with each inhibitor. To further assess effects of the inhibitors on *P. tabacina*, spore suspensions were made using the aforementioned concentrations of each inhibitor. These were used to drop inoculate several *N. obtusifolia* plants of both accessions, and symptom progression and sporulation were recorded. These experiments were repeated twice for all inhibitors and water (totaling 30 treatments for each inhibitor and water). Data were subject to statistical analysis using the  $\chi^2$  test, least significant difference, and Tukey's test.

**Microscopic analysis.** Tissue samples were taken from both *P. tabacina*-inoculated and mock-inoculated (both by infiltration) plants of accessions PI43 and PI73 each day beginning 3 dpi until the necrotic response was observed on accession PI73 (6 dpi). Prior to cutting, leaves were placed in 15-by-100-mm petri plates in which a 1- to 2-mm-thick piece of wax was placed ( $\approx 5$  cm<sup>2</sup>, to facilitate cutting without damaging tissue) and the leaves were covered in prefixing solution: 3% glutaraldehyde in 0.1 M cacodylate buffer (1.8 ml of 25% glutaraldehyde, 7.5 ml of 0.2 M cacodylate buffer [pH 7.2], and 5.7 ml of water). Five pieces (1 to 2 mm<sup>2</sup>) were cut from each of eight inoculation sites (40 pieces total for each accession on each of days 3 to 6 postinoculation). The same number was similarly cut from mock-inoculated controls. Samples then were placed into small glass containers one-third filled with prefixing solution and refrigerated overnight. Prefixing solution was removed gently with a Pasteur pipette and samples were rinsed three times with 0.2 M cacodylate buffer. For the third rinse, samples were agitated gently for 10 min on a shaker. The containers then were one-third filled with 0.2 M cacodylate buffer and one-third with 2% OsO<sub>4</sub> (osmium tetroxide; 1:1 ratio) and the samples were allowed to fix for 1 h at 4°C. Samples then were washed with distilled water five times to remove excess OsO<sub>4</sub>.

Tissue samples were dehydrated by gentle shaking in the following solutions (15 min each): 30% ethanol (with two to three changes), 50% ethanol, 70% ethanol, twice in 90% ethanol, four times in 100% ethanol, once in 1:1 (vol/vol) ethanol:acetone for 10 min, and twice in 100% acetone for 5 min. The 100% acetone was replaced with a 2:1 mixture of 100% acetone and Spurr embedding medium (46), and the samples were shaken gently for 1 h. This mixture was replaced with a 1:2 mixture of acetone and Spurr medium and shaken for 1 h. The containers then were filled almost to the top with additional Spurr medium and stored overnight at 4°C.

Prior to embedding, the samples were allowed to reach room temperature before opening to avoid introducing moisture from the air. Flat embedding molds were filled with Spurr medium and the samples were gently transferred to molds and arranged five to seven pieces/mold. Molds then were placed into a drying oven at 70°C overnight.

Thin sections ( $\approx 1$   $\mu$ m thick) were cut from embedded samples using a microtome, heat fixed onto glass slides, stained with toluidine blue for 1 to 2 min while agitating over a flame, rinsed and air dried, and examined using a compound microscope. At least 250 thin sections were examined from each infected and control accession for days 3 to 6 postinoculation.

In addition to microscopic examination of thin sections, at least 15 pieces of tissue (1 cm in diameter) were taken from infection sites on each accession on days 3 to 6 postinoculation and were cleared and stained according to Shipton and Brown (45). Control tissue was prepared similarly. Tissues were examined for *P. tabacina* colonization using compound and dissecting microscopes.

**Genetic segregation.** Several flowers on one plant of accession PI73 were emasculated 1 day prior to opening. Pollen of accession PI43 was applied to each stigma 1 day later and the plant was maintained in a greenhouse until seed harvest (22 days). Flowers on a single F<sub>1</sub> plant from this cross then were self-pollinated to produce F<sub>1</sub> seed. In all, 110 F<sub>2</sub> plants, along with 5 plants each of

TABLE 2. Geographic origins of *Nicotiana obtusifolia* accessions

Accession	Geographic origin, source
PI#555543	Unknown; United States Department of Agriculture (USDA) Tobacco Collection
PI#555573	Unknown; USDA Tobacco Collection
AusTRC303759	Mexico; Australian Tropical Crops & Forages Genetic Resource Centre; (obtained from Japan Tobacco, Inc.)
AusTRC314549	Not given; Australian Tropical Crops & Forages Genetic Resource Centre
NIC439	Unknown; Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK) Gatersleben (obtained from the Botanical Garden of the University of Birmingham, England)
NIC455	Guanajuato, Mexico; IPK-Gatersleben (obtained from Berlin Botanical Garden)
NIC456	Unknown; IPK (obtained from Toulouse, France)
DZ02-001	Desert Botanical Garden (DBG), Phoenix, AZ; shade house
DZ02-002	DBG, Phoenix, AZ; "Australian" area
DZ02-004	DBG, Phoenix, AZ; sand bed
DZ02-005	Tonto National Forest, near Payson, Gila Co., AZ
DZ02-007	Dry sandy wash south of Gillespie Dam, north of Gila Bend, Maricopa Co., AZ; Old US highway 80, near intersection with Patterson Rd
DZ02-008	Highway 86, east of Why, AZ between mile markers 59 & 60
DZ02-009	Same as DZ02-008
DZ02-010	Same as DZ02-008
DZ02-011	Same as DZ02-008
DZ02-012	At intersection of Highways 86 & 286 in Robles Junction, Pima Co., AZ
DZ02-013	Tucson, AZ; E. Broadway behind Albertson's supermarket
DZ02-014	Tucson, AZ; E. Broadway, near intersection with Bonanza Ave., N. side of road
DZ02-015	Arizona-Sonora Desert Museum, west of Tucson, AZ
DZ02-016	Dry wash (Ash Creek) off Highway 188 NW of Roosevelt Lake in Gila County, AZ (seed from previous season's growth)
DZ02-017	Beside northbound lanes of Highway 87 NE of Fountain Hills, Maricopa Co., AZ
DZ02-018	DBG, Phoenix, AZ; area known as the "minifarm"
DZ02-019	DBG, Phoenix, AZ; minifarm
DZ02-020	DBG, Phoenix, AZ; minifarm (flowers have long corollas)
DZ02-021	DBG, Phoenix, AZ; "Australian" area (flowers have short corollas)
DZ02-022	Garden of the lodge "El Oeste", Phoenix, AZ
DZ02-023	Yard of house on Freeman Rd., east side of Tucson, AZ; gift of Charlie Kane
DV#1	Death Valley National Park (DVNP), CA; Johnson Canyon in Panamint Mountains, elevation 1,290 m; gift of Dana York, DVNP botanist
DV#2	Death Valley National Park, CA; Nevares Springs area in the Funeral Mountains, elevation 255 m; gift of Dana York, DVNP botanist
DV#3	Same as DV#2, less than 100 m away; gift of Dana York
BBNP (all)	Big Bend National Park (BBNP), TX within 1 mile of park headquarters; gift of Joe Sirotak, BBNP botanist

the two parental lines and the  $F_1$ , were drop inoculated with sporangia of *P. tabacina* KY79 at 42 days of age. All plants for this experiment were grown together under identical conditions in the greenhouse and were conditioned in the inoculation chamber for 5 days prior to inoculation. Inoculated plants were maintained as described above, and inoculated leaves were removed and placed in conditions favoring pathogen sporulation as described above for maintenance of *P. tabacina* isolates. Symptoms of chlorosis, necrosis, and the relative level of sporulation were recorded for each plant.

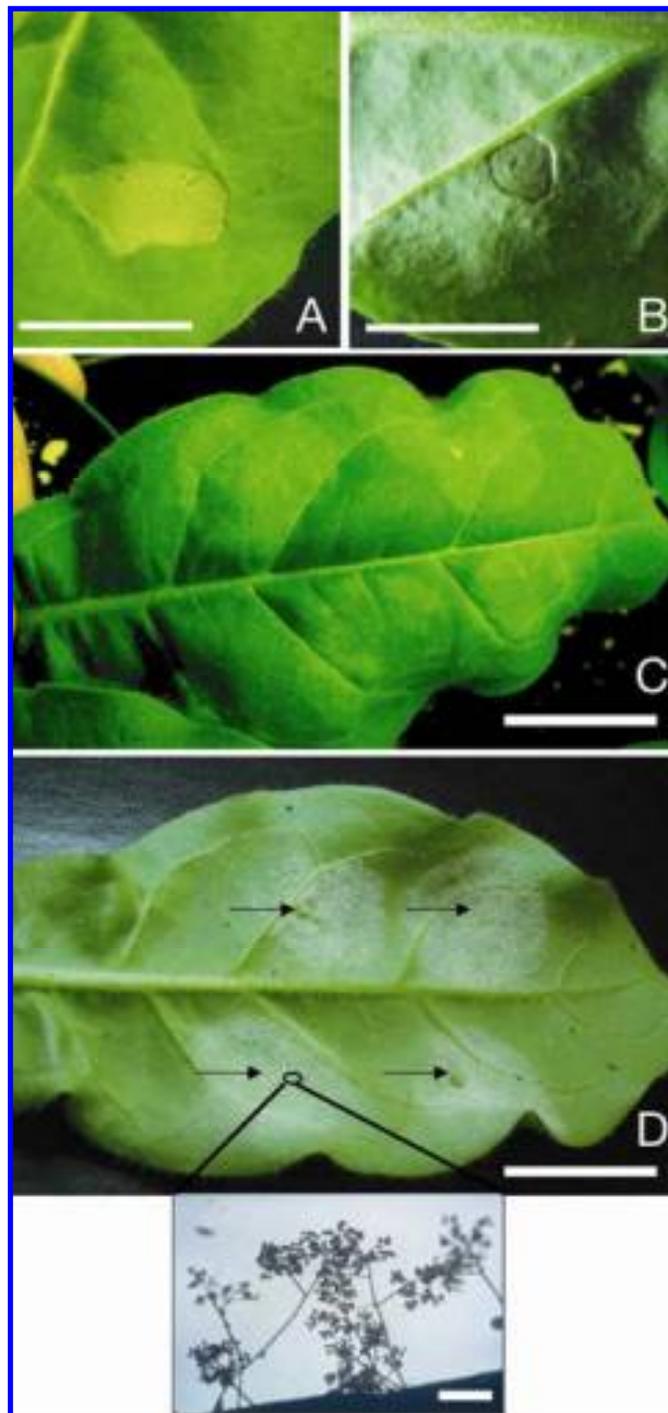
**Nucleic acid manipulations.** Cellular RNA was prepared from young leaves of inoculated plants using the Qiagen RNeasy Plant Mini kit (Qiagen Inc., Valencia, CA). RNA concentration was determined spectrophotometrically, and 5- $\mu$ g samples were electrophoresed in 1.2% (wt/vol) agarose gels containing 1 M formaldehyde. Fractionated RNA was transferred to Nytran N membrane (Schleicher & Schuell Inc., Keene, NH) in 10 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl and 0.015 M sodium citrate) using a pressure blotter (Stratagene, Inc., La Jolla, CA), and the RNA was crosslinked to the membrane with UV light. A region of the *PR-1a* gene was amplified directly from mRNA of *N. tabacum* cv. KY14 via reverse transcription PCR (OneStep RT PCR kit; Qiagen Inc., Valencia, CA) using primers *PR-1upper* (5'-CTATGCGCAAATTATGCTTCC-3') and *PR-1lower* (5'-TTTCCCCTTAATTATGACCACTTG-3') (GenBank accession no. X12737, positions 1120 to 1616) (40). The 496-bp DNA fragment was labeled to high specific activity with  $^{32}$ P- $\alpha$ -dCTP (6,000 Ci/mmol, ICN) by using the Prime-It II kit (Stratagene). Northern blot hybridization was carried out in 7% sodium dodecyl sulfate (SDS, 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA supplemented with sheared herring-sperm DNA (100  $\mu$ g/ml; Promega, Madison, WI), and powdered milk (1 mg/ml) for 16 h at 65°C in a rotary hybridization oven. Membranes were washed twice in 1 $\times$  SSC, 0.1% SDS for 10 min, first at room temperature and then at 65°C, and finally in 0.5 $\times$  SSC, 0.1% SDS for 30 min at 65°C. Images were obtained using a Molecular Dynamics PhosphorImager 445 SI instrument (Molecular Dynamics, Sunnyvale, CA), and the bands were background-corrected and quantified using ImageQuant software (Molecular Dynamics). Band intensities of the 26S rRNA, taken from the gel image, were background corrected and quantified using Kodak 1D Image Analysis software (Eastman Kodak, Rochester, NY). These values then were used to normalize the *PR-1a*-specific hybridization signals to correct for lane-to-lane variation in RNA loading.

## RESULTS

**Responses of *N. obtusifolia* accessions to *P. tabacina* infections.** Drop inoculation of 45-day-old plants of *N. obtusifolia* PI73 with viable *P. tabacina* sporangia resulted in the appearance of localized necrotic lesions 1 to 1.5 cm in diameter within 5 to 6 dpi. The earliest signs of tissue collapse were noted 1 day earlier as small, green, papery areas at the original inoculation sites (Fig. 1A). These initial lesions underwent expansion and became tan or brown 12 to 24 h later (Fig. 1B). Chlorosis was not observed on PI73 plants inoculated with *P. tabacina*. Induction of sporulation at 8 dpi on PI73 plants resulted in either no observable sporulation (determined by microscopy), scant sporulation (2 or 3 sporangiophores), or, rarely, small patches of sporangiophores 1 to 2 mm in diameter on asymptomatic leaf tissue around the margins of the necrotic lesions. Of the inoculation sites, 15% ( $n = 480$ ) did not develop necrosis or any other symptoms and did not support sporulation of the pathogen following induction, and thus were termed "misses."

The response of accession PI43 to *P. tabacina* infection differed markedly from that of accession PI73. Infected PI43 plants developed circular chlorotic lesions that were readily visible beginning at 5 dpi. These lesions expanded to form larger circular le-

sions 2 to 3 cm in diameter with more intense chlorosis developing at 6 to 10 dpi (Fig. 1C). A small number of lesions (percentage not determined) had a 2- to 3-mm necrotic center surrounded by chlorotic tissue (data not shown). Inducing sporulation at 8 dpi resulted in abundant production of sporangiophores on infected plants of accession PI43 (Fig. 1D), giving the velvety-gray, downy appearance characteristic of tobacco blue mold. Sporulation was observed on leaf tissue that was chlorotic, on surrounding asymptomatic regions (extending at least 1 cm beyond the chlorosis),



**Fig. 1.** Response of *Nicotiana obtusifolia* PI73 to foliar infection by *Peronospora tabacina*: **A**, Initial necrotic collapse at 5 days postinoculation (dpi); **B**, hypersensitive response at 6 dpi; **C**, chlorotic lesions on leaf of *N. obtusifolia* PI43 at 5 dpi; **D**, areas of *P. tabacina* sporulation (arrows) on abaxial surface of infected leaf from panel C at 7 dpi. Bars equal 3 cm. Thumbnail image shows close-up of *P. tabacina* sporangiophores bearing asexual sporangia. Bar equals 100  $\mu$ m.

and, occasionally, over the entire inoculated leaf. Of the inoculation sites on accession PI43 plants, 12% ( $n = 480$ ) resulted in misses.

To test whether the necrosis that developed on inoculated PI73 plants could have been a response to microbial contaminants in the *P. tabacina* inoculum, plants were inoculated with *P. tabacina* sporangia collected from *N. repanda* grown in gnotobiotic associations. Necrosis and chlorosis, similar to that observed in previous experiments, developed on PI73 and PI43 plants, respectively. Thus, the induction of necrotic lesions was most likely due to *P. tabacina* infection, and not from interactions with contaminating microbes in the inoculum.

Disease progression and host responses were characteristic of the *N. obtusifolia* accessions and were independent of the inoculation method (drop or infiltration), pathogen isolate, or source of inoculum (*N. tabacum* cv. KY14 grown in the growth chamber or *N. repanda* in gnotobiotic associations).

**Effect of plant age.** Experiments were performed to determine whether plant age affected host response to *P. tabacina* infection. All PI73 plants, 3 weeks old or younger, developed chlorotic lesions within 6 dpi. Because plants less than 2 weeks old were small (some leaves were only 5 to 10 mm across), separate lesions were not discernable. Abundant sporulation occurred on these plants following induction and the sporangia were used to com-

plete modified Koch's postulates. Among the PI73 plants inoculated at 4 weeks of age, 4 of 20 (20%) exhibited tissue collapse and necrosis, while the rest developed chlorotic symptoms with associated pathogen sporulation. Four-week-old PI73 plants that developed necrosis supported some pathogen sporulation on asymptomatic tissue around the edges of the necrotic lesions similar to what was observed with infected PI73 plants in previous experiments. Plants of accession PI73, inoculated at 5 weeks of age or older, displayed tissue collapse and necrosis on all inoculation sites where infection occurred. Plants that were 7 weeks old were observed to have the most misses (average of 32%) compared with 5- and 6-week-old plants (average of 12 and 17%, respectively;  $n = 20$  in each case). At 7 weeks of age, some plants had begun to develop an inflorescence, as evidenced by elongation of the main stem (bolting).

Inoculation of PI43 plants with *P. tabacina* sporangia resulted in production of chlorotic lesions, regardless of age. Some chlorotic lesions produced on PI43 plants at or beyond 3 weeks of age had small necrotic centers (2 to 3 mm). Induction of sporulation on PI43 and PI73 plants was as observed in previous experiments. There was little or no sporulation around the margin of the necrotic lesions on PI73 plants, but abundant sporulation on chlorotic lesions of PI43 plants that often extended 1 cm or more into asymptomatic tissue or covered the entire surface of the inoculated leaf.

**Timing of sporulation.** Subsets of *N. obtusifolia* PI43 and PI73 plants were placed into conditions conducive to *P. tabacina* sporulation each day after inoculation (plants were 45 days old at inoculation). No sporulation was observed on infection sites of PI73 plants until the first sign of tissue collapse was observed (typically 5 dpi). Moreover, no sporulation was observed on PI43 plants until the first signs of collapse appeared on infected PI73 plants. Sporulation on PI73 plants extended up to 1 cm beyond the area of tissue collapse when induced the first day on which collapse occurred (5 to 6 dpi). On subsequent days, sporulation either was not observed on PI73 plants or was scant, as described previously.

**Effects of metabolic inhibitors.** Treatment of *P. tabacina*-inoculated PI73 plants with 0.5 mM  $\text{CoCl}_2$  resulted in complete inhibition of necrosis (Fig. 2), except for a total of four spots (on different plants across three experiments) that developed a patchy necrosis in the zone where the inhibitor was infiltrated (Table 3). Treatment of inoculated PI73 plants with 50  $\mu\text{M}$  sodium orthovanadate typically resulted in a patchy necrosis, suggesting intermediate or partial inhibition of the necrotic response. Sodium orthovanadate did not noticeably affect uninoculated controls (Table 3). Infected tissues of PI73 plants were treated with 5  $\mu\text{M}$  cycloheximide, an 80S ribosome inhibitor, at 4 dpi, with no discernible effect on the subsequent necrotic response (Table 3). However, cycloheximide treatment resulted in localized necrosis of the leaf tissue surrounding all zones of infiltration on inoculated plants and uninoculated controls (indicative of phytotoxicity).



**Fig. 2.** *Nicotiana obtusifolia* PI73 leaves inoculated with *Peronospora tabacina* (left) or mock-inoculated with water (right), shown at 6 days post-inoculation (dpi). Lesions were treated either with  $\text{CoCl}_2$  (white arrows) or water (black arrows) at 4 dpi. Note that the  $\text{CoCl}_2$  treatments greatly reduced the size of the area showing necrosis. Bar equals 3 cm.

TABLE 3. Results of treatment with three different metabolic inhibitors and water on development of the necrotic response on leaves of *Nicotiana obtusifolia* PI73

Treatment <sup>y</sup>	Concentration (M)	Response <sup>z</sup>			
		Positive	Intermediate	Negative	Miss
Cobalt chloride	$5 \times 10^{-4}$	0.0 b	1.3 b	<u>6.7 a</u>	2.0 a
Orthovanadate	$5 \times 10^{-5}$	2.3 b	<u>6.0 a</u>	0.3 b	1.3 a
Cycloheximide	$5 \times 10^{-6}$	<u>8.0 a</u>	0.0 b	0.0 b	2.0 a
No inhibitor (water)	...	<u>7.7 a</u>	0.0 b	0.0 b	2.3 a
LSD ( $P = 0.0001$ )	...	2.7	2.3	1.5	3.8

<sup>y</sup> Inhibitor solution or water was infiltrated into the leaf 4 days after inoculating with sporangia of *Peronospora tabacina* KY79.

<sup>z</sup> In each of three experiments, phenotypes of 10 inoculation sites for each treatment were scored as follows: Positive = collapse and necrosis of the tissue following treatment; Intermediate = only patchy necrosis; Negative = absence of necrotic response in the zone where inhibitor was infiltrated; Miss = inoculation site failed to develop necrosis by day 5, and also failed to exhibit signs of the pathogen in the subsequent attempt to induce sporulation. Shown is the number of lesions exhibiting each phenotype, averaged across the three experiments. Mean values within a response column followed by different letters are significantly different at  $P = 0.0001$  as determined Tukey's Test or least significant difference (LSD). Underlined values are significantly different from other values within a treatment row ( $\chi^2$ ,  $P < 0.0001$ ).

Following induction, the pathogen sporulated on infection sites treated with  $\text{CoCl}_2$  or sodium orthovanadate, regardless of plant accession. Sporulation occurred similarly on inhibitor-treated inoculation sites compared with those treated with water, with the exception of water-treated PI73 plants on which necrosis occurred and sporulation was scant. Sporangia were collected from plants of both accessions that were treated with inhibitors, and were used to complete modified Koch's postulates.

Sporangial suspensions containing concentrations of  $\text{CoCl}_2$  required to block the necrotic response were used to drop inoculate both resistant and susceptible plant accessions. These treatments resulted in infection followed by chlorotic (PI43 plants) or necrotic (PI73 plants) host symptoms 5 to 6 dpi, and did not differ from treatments with sporangia alone with respect to timing of symptom development and pathogen sporulation. Moreover, sporangia that were suspended in 5 mM  $\text{CoCl}_2$ , a concentration 10-fold higher than was required to inhibit necrosis on PI73 plants, also were capable of infecting either *N. obtusifolia* accession.

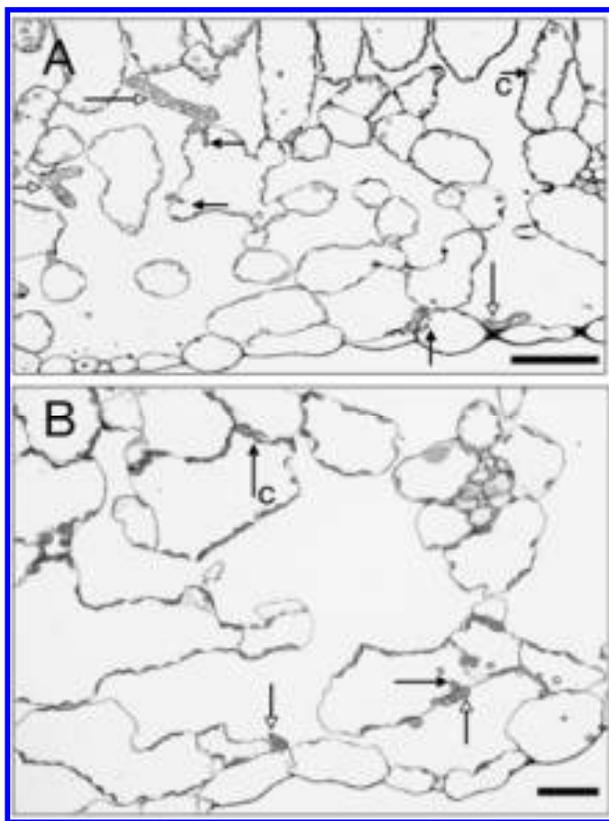
**Microscopic observations.** Intercellular pathogen hyphae and haustoria were evident at 3 dpi in inoculated plants of accessions PI43 and PI73 (Fig. 3A and B). Collapse of individual cells in contact with haustoria was not observed at 3 or 4 dpi in tissues from either host accession. Examination of thin sections from PI73 plants at 5 dpi revealed complete disorganization of the cells associated with areas of visible collapse (Fig. 4A). Specifically, cell walls appeared thinner and wavy or ribbon-like and were completely disorganized compared with those observed on previous days. Inter- and intracellular spaces were barely distinguishable from one another, and palisade cells were not discernible from mesophyll cells as they had been 1 day prior. In addition, chloroplasts that were easily identified 1 day prior (Fig. 3A and B) were not evident following collapse (Fig. 4A). Examination of thin sections of PI73 plants at 6 dpi (necrotic tissue) revealed an

even greater degree of disorganization and thinning of the leaf tissue compared with previous days (data not shown). Conversely, at 5 and 6 dpi, the cells of accession PI43 plants were organized similarly to those observed at 3 and 4 dpi, except that pathogen hyphae were more abundant (Fig. 4B) and no cellular disorganization was observed.

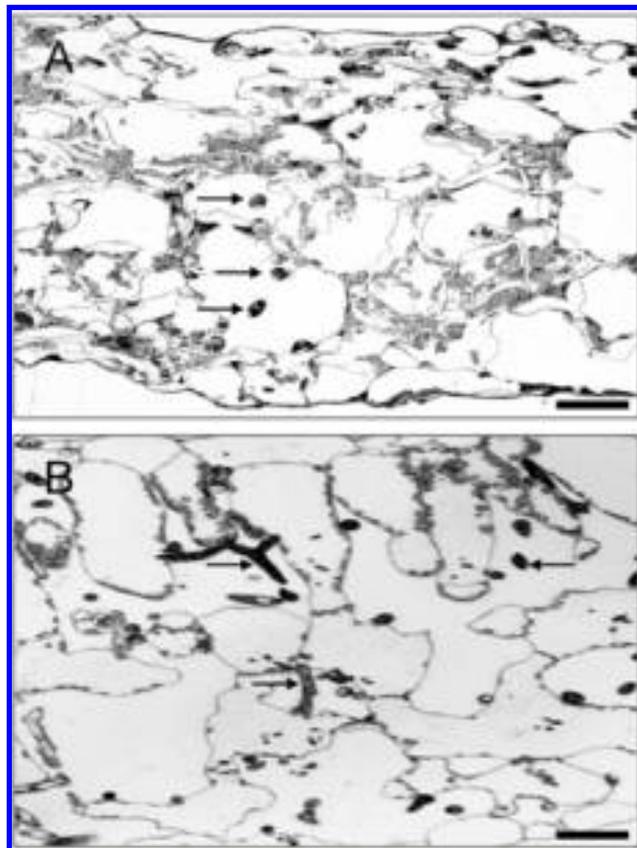
Examination of tissue pieces from the two accessions revealed little or no differences with respect to the degree of pathogen colonization from 1 to 4 dpi. Following tissue collapse on PI73 plants at 5 dpi, hyphae were not as easily recognizable as they had been in tissue from the previous days. For this reason, further comparisons between PI43 and PI73 plants could not be made regarding pathogen colonization or changes in the host tissue.

**PR gene expression.** Northern blots containing RNA extracted from inoculated leaves of PI73, PI43, and  $F_1$  (PI73  $\times$  PI43) plants at 1, 2, 3, 4, and 5 dpi were subjected to hybridization with radiolabeled *PR-1a* cDNA from *N. tabacum* (Fig. 5). RNA from mock (water)-inoculated plants was included as a control. *PR-1a*-specific mRNA was detectable at 2 dpi in blue mold-inoculated PI73 plants, and accumulated to its highest level at 5 dpi. In both PI43 and  $F_1$  plants, however, *PR-1a* gene mRNA was not detectable until 5 dpi. *PR-1a* gene-specific mRNA was not detected in any of the water-inoculated controls (Fig. 5A). Band densities of the ethidium bromide-stained 26S rRNA were used to normalize the expression of the *PR-1a* gene mRNA (to account for variation in the amount of RNA loaded per lane), and the mRNA abundance was plotted against days postinoculation for the two parental lines and the  $F_1$  hybrid. The results (Fig. 5B) clearly show that transcription of the *PR-1a* gene is rapidly induced in PI73 plants upon infection with *P. tabacina*, but that this effect is delayed in blue mold-infected PI43 plants and the  $F_1$  hybrid.

**Survey of *N. obtusifolia* accessions.** Four *N. obtusifolia* accessions, three from Arizona (DZ02-004, DZ02-007, and DZ02-017)



**Fig. 3.** Micrographs of **A**, *Nicotiana obtusifolia* PI73 and **B**, PI43 4 days after inoculation with *Peronospora tabacina*. Black arrows indicate haustoria, arrows with unfilled heads indicate intercellular hyphae, and arrows labeled "C" indicate chloroplasts. Bars equal 50  $\mu\text{m}$ .



**Fig. 4.** Micrographs of **A**, *Nicotiana obtusifolia* PI73 and **B**, PI43 5 days after infection with *Peronospora tabacina*. Black arrows indicate intercellular hyphae. Bars equal 50  $\mu\text{m}$ .

and one from Mexico (303759), were characterized as being similar to PI73 in their responses to inoculation with *P. tabacina* (necrosis without chlorosis or subsequent sporulation). Four other accessions from southern Arizona developed necrotic lesions without chlorosis in response to *P. tabacina* infection (Table 4). Because a few sporangiophores were observed at the lesion margins on these plants, they were given sporulation ratings of 1.5. Accessions DZ02-020, 314549, and DV#2 were rated as being similar to USDA accession PI43 in exhibiting chlorotic symptoms without necrosis and with heavy sporulation following induction (Table 4). All other accessions gave a foliar response consisting of zones of necrosis surrounded by chlorosis. In these plants, the chlorotic tissue supported heavy pathogen sporulation, sometimes involving both leaf surfaces (sporulation ratings of 3 to 4) (Table 4). Necrotic lesions on plants that gave an intermediate response continued to expand until 8 dpi, sometimes causing collapse of the entire leaf, whereas necrotic lesions on PI73, DZ02-004, DZ02-007, and DZ02-017 plants were confined to the size of the initial area of collapse. PI43 and PI73 plants that served as controls developed symptoms consistent with those described for previous experiments.

**Genetic segregation.** In all, 110 F<sub>2</sub> plants from the cross PI73 × PI43 were inoculated with sporangia of *P. tabacina* KY79. Based on comparisons with inoculated PI43, PI73, and F<sub>1</sub> plants at 8 dpi, the F<sub>2</sub> plants were scored as having (i) necrosis only (similar to PI73), (ii) necrosis with associated chlorosis (similar to the F<sub>1</sub> plants), or (iii) chlorosis only (similar to PI43). Disease reactions on the parental lines and F<sub>1</sub> hybrids were quite uniform. Lesions developed on one or more inoculation sites of all inoculated plants, but not on uninoculated or mock-inoculated plants. The overall percentage of successful inoculation sites was 56% (no evidence of infection in the other 44%), and 104 of the 110 plants could be scored without difficulty. All disease ratings were

confirmed by inducing pathogen sporulation on infected leaves at 8 dpi. Sporulation was absent on plants rated as showing only necrosis, heavy on those rated as having only chlorosis, and moderate to heavy on those in the necrosis + chlorosis class.

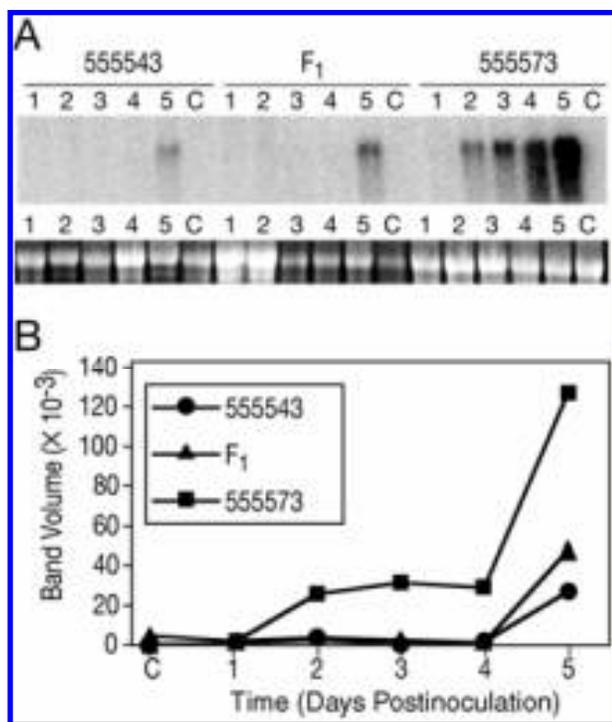
The F<sub>2</sub> progeny plants were scored as follows: necrosis only, 28; necrosis + chlorosis, 66; and chlorosis only, 10. Expected values for the segregation of a single gene for resistance in an F<sub>2</sub> population would be 78 necrosis, 26 chlorosis (3:1) if resistance was fully dominant; 78 chlorosis, 26 necrosis (3:1) if resistance was recessive; and 26 necrosis, 52 necrosis + chlorosis, and 26 chlorosis (1:2:1) if the R allele showed incomplete dominance. The genetic segregation data were tested for significance using the  $\chi^2$  statistic. We found that the incomplete dominance model for inheritance of a single gene was not supported ( $P = 0.012$ ), probably due to misclassification of some of the susceptible F<sub>2</sub> plants (described below). When the plants were rescored as either resistant (R) (necrotic lesions with no inducible *P. tabacina* sporulation) or susceptible (S) (chlorotic lesions with or without accompanying necrosis, with moderate to heavy pathogen sporulation), the analysis supported the 1:3 (R:S) pattern of inheritance ( $P = 0.75$ ). Based on these disease ratings (symptom development and pathogen sporulation), we conclude that blue mold resistance in *N. obtusifolia* PI73 appears to be due to the action of a single gene, which we have named *Rpt1*. Because heterozygous F<sub>1</sub> plants displayed an intermediate phenotype when infected by *P. tabacina*, we consider *Rpt1* to be partially dominant rather than recessive in its action.

## DISCUSSION

The main objective of this study was to determine whether the necrotic response observed on *N. obtusifolia* PI73 following *P. tabacina* infection is a manifestation of the HR that occurs during incompatible plant–pathogen interactions, and to determine the underlying genetics of the response. Among other *Nicotiana* spp., only *N. exigua* H.-M. Wheeler has been shown to develop an HR-like reaction to *P. tabacina* infection (3); lesions appear 2 dpi, but involve only cells on the adaxial surface of the leaf. However, the genetic and mechanistic basis of the *N. exigua* response is unknown. Here we present evidence that the *N. obtusifolia* response is an HR governed by a single, partially dominant gene, and involving plant signal transduction.

Because the HR elicited by several plant pathogens is an active response, dependent upon the activation of host transcriptional machinery it can be blocked by chemical inhibitors of host metabolism and signal transduction (22,26). CoCl<sub>2</sub>, which blocks influx of Ca<sup>2+</sup> across the plasma membrane, eliminated the HR in *P. tabacina*-infected *N. obtusifolia* PI73. CoCl<sub>2</sub> previously has been shown to suppress bacterially mediated hypersensitive cell death in suspension-cultured tobacco cells (1). Co-infiltration of the inhibitors with inoculum had little or no effect on the pathogen, indicating that HR suppression by CoCl<sub>2</sub> and, to a lesser degree, sodium orthovanadate was most likely due to effects on host metabolism or signaling. Thus, the response appeared similar to HRs in other pathosystems.

Leaf-cell disorganization, another hallmark of the HR (12,29) typically occurred 4 to 5 dpi in plants of *N. obtusifolia* PI73 infected with *P. tabacina*, but not in infected PI43 (susceptible) plants. Appearance of the necrotic response (5 to 6 dpi) on inoculated plants of *N. obtusifolia* PI73 coincided in time with the pathogen's ability to produce asexual sporangia. However, sporulation was not required for the development of necrosis. It is possible that the necrotic response results from interaction of the host with an elicitor molecule (possibly a protein) produced by the pathogen at or before the developmental stage (or age) in which it is capable of sporulating. Small proteinaceous elicitors (elicitins) have been characterized in *Phytophthora* and *Pythium* spp., other oomycetous pathogens of plants



**Fig. 5.** Transcription of the *PR-1a* gene ortholog in *Nicotiana obtusifolia* infected with *Peronospora tabacina* at 1, 2, 3, 4, and 5 days postinoculation. **A**, Northern blot of total RNA isolated from leaves of *N. obtusifolia* probed with a region of the tobacco *PR-1a* gene (upper) and EtBr-stained 26S rRNA bands from the agarose/formaldehyde gel (lower). **B**, Numerical data from **A** displayed graphically. Band volumes (an arbitrary measure of band intensity) from the northern blot were normalized using the 26S rRNA band. Water-inoculated controls are designated “C”.

(35,38), and some of these molecules have been shown to bind phytosterols (32).

The necrotic response may imply a gene-for-gene or race-specific relationship (16,17,37) in the *Peronospora tabacina*-*N. obtusifolia* interaction. Determining the underlying genetics of the pathogen is not possible at present because *P. tabacina* is not known to have a fully functional sexual stage. However, the pattern of inheritance of the necrotic response phenotype in the host suggested major involvement of a single plant gene (*Rpt1*) that displayed incomplete dominance. Of considerable interest is the relationship between the expression of HR-mediated blue mold resistance and developmental stage in PI73 plants. We found that, in order for *N. obtusifolia* PI73 to consistently express the necrotic lesion response, plants needed to be at least 5 weeks old. Genes other than *Rpt1* that are expressed at >5 weeks of age could be necessary for full expression of the HR, and isolation and characterization of these genes may contribute to the broader understanding of HR-mediated disease resistance in plants. A dependence on host plant age as it relates to *P. tabacina* resistance

was noted by Clayton (8) for several species of *Nicotiana*, although this type of resistance is fundamentally different from what we observed in *N. obtusifolia* PI73.

The results of the genetic segregation experiment were clear, despite difficulties encountered in scoring a subset of the susceptible plants; those that developed chlorotic lesions with the smallest amount of necrosis were assigned to the chlorotic + necrotic (intermediate) class, even though some of these supported heavy pathogen sporulation following induction. Thus, some plants that were genotypically homozygous for the susceptible allele (*rpt1/rpt1*) must have been classified phenotypically as heterozygous. This can be explained by the fact that the parents of the segregating population are quite different morphologically, and novel genetic combinations due to segregation of many uncharacterized genes potentially could influence the reaction of progeny plants to infection by the blue mold pathogen. We noticed that traits such as time to flowering and plant size varied within the group of F<sub>2</sub> progeny; both of these characters are components of plant maturity, which is known to play a role in the

TABLE 4. Tobacco blue mold disease ratings for *Nicotiana obtusifolia* accessions used in this study

KTRDC accession no. <sup>†</sup>	Alternative designation <sup>‡</sup>	Disease symptoms <sup>‡</sup>	Sporulation rating <sup>‡</sup>	Subjective rating <sup>‡</sup>
PI#555543 <sup>‡</sup>	TW 98	C, N+C	3.5	Highly susceptible
PI#555573 <sup>‡</sup>	TW 143	N	1	Highly resistant; HR
None	314549 <sup>‡</sup>	N+C	4	Highly susceptible
S-10-5	303759 <sup>‡</sup>	N	1	Highly resistant; HR
S-10-6	DZ02-001	N+C	3.5	Highly susceptible
S-10-7	DZ02-002	N+C	3.5	Highly susceptible
S-10-8	DZ02-004	N	1	Highly resistant; HR
S-10-9	DZ02-005	N	2.5	Susceptible (low level of infection)
S-10-10	DZ02-007	N, N+C	1	Highly resistant; HR
S-10-11	DZ02-008	N+C	2	Moderately resistant
S-10-12	DZ02-009	N+C	2	Moderately resistant
S-10-13	DZ02-010	N, N+C	2	Moderately resistant
S-10-14	DZ02-011	N+C	2.5	Susceptible
S-10-15	DZ02-012	N+C	1.5	Resistant; HR
S-10-16	DZ02-013	N+C	2.5	Susceptible
S-10-17	DZ02-014	N, N+C	1.5	Resistant; HR
S-10-18	DZ02-015	N+C	2.5	Susceptible
S-10-19	DZ02-016	N+C	3.5	Highly susceptible
S-10-20	DZ02-017	N	1	Highly resistant; HR
S-10-21	DZ02-018	N+C	2	Moderately resistant
S-10-22	DZ02-019	N, N+C	4	Highly susceptible
S-10-23	DZ02-020	C	4	Highly susceptible
S-10-24	DZ02-021	N	1.5	Resistant; HR
S-10-25	DZ02-022	N	1.5	Resistant; HR
S-10-26	DV#1	N+C	4	Highly susceptible
S-10-27	DV#2	C	4	Highly susceptible
S-10-28	DV#3	N+C	2.5	Susceptible
S-10-29	BBNP-1	N+C	4	Highly susceptible (heavy chlorosis)
S-10-30	BBNP-2	N+C	4	Highly susceptible (heavy chlorosis)
S-10-31	BBNP-3	N	2.5	Susceptible
S-10-32	BBNP-4	N+C	4	Highly susceptible
S-10-33	BBNP-5	C, N+C	4	Highly susceptible (heavy chlorosis)
S-10-34	BBNP-6	N+C	4	Highly susceptible (heavy chlorosis)
S-10-35	BBNP-7	N+C	4	Highly susceptible (heavy chlorosis)
S-10-36	BBNP-8	N+C	4	Highly susceptible (heavy chlorosis)
S-10-37	BBNP-9	N+C	4	Highly susceptible
S-10-38	BBNP-10	N+C	3.5	Highly susceptible
S-10-39	BBNP-11	N+C	3.5	Highly susceptible
S-10-40	BBNP-12	N, N+C	2.5	Susceptible
S-10-41	DZ02-023	N+C	3.5	Highly susceptible
S-10-42	NIC439 <sup>‡</sup>	C, N+C	4	Highly susceptible
S-10-43	NIC455 <sup>‡</sup>	N+C	4	Highly susceptible
S-10-44	NIC456 <sup>‡</sup>	C, N+C	4	Highly susceptible

<sup>†</sup> KTRDC = Kentucky Tobacco Research and Development Center, University of Kentucky.

<sup>‡</sup> Original accession number or field collection number.

<sup>‡</sup> Ratings of five individuals, 12 inoculation sites/plant. N = necrosis only, C = chlorosis only, and N+C = necrosis and chlorosis; assayed at least twice.

<sup>‡</sup> Subjective rating scale, where 1 = no visible sporulation, 2 = small patches of sporangioophores present around the lesion margin, 3 = zone of sporulation extending up to 1 cm outward from the lesion border, and 4 = heavy sporulation encompassing the entire symptomatic area or leaf surface.

<sup>‡</sup> HR = expresses hypersensitive response to *P. tabacina* infection.

<sup>‡</sup> Originally *N. palmeri*.

<sup>‡</sup> Originally *N. trigonophylla*.

development of blue mold lesions in *N. obtusifolia* (this study) and other species of *Nicotiana* (42). Of interest here is the observation by Holub et al. (27) that an allele of *RPP1* from *Arabidopsis thaliana* ecotype Nd-1 displayed incomplete dominance in crosses between Nd-1 and either Col-5, Ler-1, or Oy-1, but was completely dominant in a cross with ecotype Wei-1 when the F<sub>2</sub> progeny were inoculated with *P. parasitica* Emoy2. Similarly, lettuce (*Lactuca sativa*) plants heterozygous at the *R6* locus for resistance to *Bremia lactucae* showed increased hyphal colonization and significantly higher infection scores when inoculated with avirulent strains of the pathogen than did homozygous plants, which was interpreted as evidence that the *R6* gene is incompletely dominant (10).

Single genes conferring resistance to oomycete pathogens related to *P. tabacina* have been well studied in lettuce and *A. thaliana* (31). Eleven characterized downy mildew resistance genes located on four linkage groups in lettuce are all apparently dominant, and provide varying degrees of race-specific resistance to *B. lactucae* pathotypes (9). In *A. thaliana*, race-specific resistance to *P. parasitica* is conferred by the *RPP* genes, which occur in the various ecotypes as either simple loci (4), complex loci (5), or multigene families (36). Plants heterozygous at either *RPP4* or *RPP5* display an intermediate resistance phenotype (partial resistance) when inoculated with *P. parasitica* races Emwal and Noco2, respectively, scored based on production of asexual spores of the pathogen (39,48). In the *N. obtusifolia*–*P. tabacina* pathosystem, we considered plants to be fully resistant only if they developed the HR and did not support pathogen sporulation.

*PR-1a*, along with other PR protein genes, is specifically induced in response to pathogen attack and other stresses in plants (44). Transcription of many PR genes is induced in response to endogenous salicylic acid (SA), which correlates with appearance of the HR in incompatible plant–pathogen interactions (34). We expected to see defense gene transcription occurring just prior to the appearance of the HR in inoculated PI73 plants. However, *PR-1a* gene-specific transcripts showed significant accumulation at 2 dpi—several days before tissue necrosis became evident—with no mRNA accumulation observed in inoculated PI43 (susceptible) plants until 5 dpi (Fig. 5A). This is very similar to what is seen in interactions between *A. thaliana* and *P. parasitica*. *A. thaliana* ecotype Ws-0 (Wassilewskija) carries a complex *RPP1* locus that confers resistance to several isolates of *P. parasitica* (5). Resistance generally takes the form of an HR (“necrotic pits”) that becomes evident 3 dpi and prevents tissue colonization and subsequent pathogen sporulation (27). Rairdan et al. (41) showed that *PR1* is rapidly induced in Ws-0 upon infection by the avirulent isolate Noco2, with a high level of expression seen by 1 dpi. However, in the compatible interaction with isolate Emco5, *PR1* mRNA is not detectable in Ws-0 leaves until 3 dpi.

As a possible explanation for the observed temporal separation between *PR-1a* gene induction and HR-mediated cell death in *P. tabacina*-infected *N. obtusifolia* PI73, one could propose the existence of two recognition phenomena occurring in this host–pathogen interaction; an early one in response to *P. tabacina* infection that could result in the induction of SA biosynthesis and the concomitant induction of PR gene expression, and a later one that occurs when the interaction switches from compatibility to incompatibility, activating a programmed cell death pathway that culminates in HR. This model may fail to account for the lack of early PR gene transcription in the susceptible genotype (PI43), although a recent article could provide a possible explanation. Grüner et al. (20) have proposed a model for transcriptional activation of *PR-1a* in Tobacco mosaic virus-infected tobacco expressing the *N* gene, in which there are two independent signal transduction pathways that mediate gene expression via a common element of the *PR-1a* gene promoter; the familiar SA-dependent pathway that is blocked in plants expressing salicylate hydroxylase (*nahG* from *Pseudomonas putida*), and a second one

that relies on uncharacterized signals produced during the HR. Expression of *PR-1a* in *Peronospora tabacina*-infected *N. obtusifolia* could be under a similar kind of dual regulatory control, where the SA-dependent pathway is active and serves to induce *PR-1a* late in the infection, when SA has accumulated to a high level, whereas the HR-dependent pathway induces the *PR-1a* promoter starting at 1 dpi in PI73, but is somehow suppressed in PI43 and the F<sub>1</sub> hybrid. A preliminary study showed that endogenous levels of bound SA became elevated in PI43, PI73, and the F<sub>1</sub> 7 days after *P. tabacina* inoculation, increasing 69-fold, 65-fold, and 24-fold, respectively, over mock-inoculated controls (E. P. Heist, unpublished data).

We speculate that the pathogen may produce an elicitor specifically as it develops the ability to sporulate, and that *Rpt1/Rpt1* plants respond to this elicitor to give a visible HR within ≈12 h of its production. Alternatively, the 3- to 4-day lag between pathogen recognition (as determined by PR gene transcription) and onset of HR could result from a low initial concentration of both the eliciting molecule and the resistance gene product (21), or from some other time-dependent step in the signaling events downstream from *Rpt1* (such as amplification of a key signaling intermediate). This second scenario implies that the appearance of the HR as the pathogen enters its asexual reproductive phase is merely coincidental, and it does not explain the sudden (as opposed to gradual) appearance of the HR over a relatively large area of the leaf surface in drop-inoculated plants.

*N. obtusifolia* plants of accessions PI43, PI73, and others often developed symptoms of systemic *P. tabacina* infection within 2 weeks after spraying the foliage with a sporangial suspension or root inoculation (E. P. Heist, unpublished observation). This observation indicates tissue specificity of *Rpt1*-mediated resistance. We believe that the ability of *N. obtusifolia* PI73 to express the HR against *P. tabacina* is specific to cells of the leaf lamina. The pathogen can gain entry into the vasculature through the roots (25) and either leaf veins or axillary buds, when the plants are spray inoculated, and occasionally when the plants are inoculated by infiltration. Root-inoculated plants of PI73 or PI43 did not show any visible signs of infection (lesions) on the roots, and generalized systemic symptoms typically developed in 2 weeks on both accessions. However, in systemically infected plants of the resistant accession (PI73), hypersensitivity was observed when the pathogen grew into the second or third leaf panel, while stems and leaf tissues at the base of the petiole remained symptomatic (chlorotic or wrinkled) but did not exhibit HR. Pathogen sporulation could be induced on non-necrotic, symptomatic leaf tissue, but sporulation was not associated with the necrotic response. This localized HR did not occur in systemically infected PI43 plants. A likely explanation for this phenomenon is that meristematic tissues (juvenile tissues) appear to respond to *P. tabacina* infection similarly to what was observed with PI73 plants that were less than 5 weeks of age. (As stated earlier, these younger PI73 plants were susceptible and exhibited chlorotic symptoms without HR.) A similar host tissue specificity in the interaction of the Guy 11 strain of *Magnaporthe grisea*, the rice blast pathogen, with barley and wheat seedlings has been characterized by Dufresne and Osborne (15).

*Rpt1* is the first major locus identified that confers resistance to *P. tabacina* in any species of *Nicotiana*. High levels of genetic resistance (or immunity) to *P. tabacina* infection have been identified in several Australian species of *Nicotiana* (8). Tobacco cultivars incorporating polygenic resistance from *N. debneyi*, *N. velutina*, and *N. goodspeedi* have been produced in Australia and the United States (42). Rufty and Main (43) found that, in resistant *N. tabacum* genotypes, *P. tabacina* induced fewer and smaller lesions, had reduced sporulation, and exhibited longer latent periods compared with susceptible cultivars. Therefore, in cultivars expressing quantitative resistance, tobacco blue mold epiphytotic would be expected to be less severe as a result of

delayed onset and an overall reduction in pathogen development. A necrotic lesion response in cultivated tobacco would be useful for impeding the spread of tobacco blue mold by greatly reducing production of sporangia, a critical control point in epidemic development. A single gene that confers resistance to *P. tabacina* (HR and suppressed sporulation) could augment the quantitative form of resistance incorporated by breeding from Australian species of *Nicotiana* (42). Thus, a strategy to isolate *Rpt1*, the tobacco blue mold resistance gene present in *N. obtusifolia* PI73, seems a good prospect for future research. Experiments are underway to “tag” the gene using molecular markers and to produce a fertile hybrid between *N. obtusifolia* PI73 and blue mold-susceptible tobacco, using methods similar to those of Chung et al. (7), for potential use in breeding.

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