Use of Specific Repetitive Sequences in *Peronospora tabacina* for the Early Detection of the Tobacco Blue Mold Pathogen

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ABSTRACT

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A 232-bp DNA sequence was obtained from random amplified polymorphic DNA of *Peronospora tabacina*, the blue mold pathogen of tobacco. This sequence had homology to *P. tabacina* DNA but not to DNA of other downy mildew or oomycetous fungi tested. The fragment was determined to be part of a repetitive DNA sequence that was ubiquitous in a worldwide collection of *P. tabacina*. Oligonucleotides were designed for amplification of this sequence by polymerase chain reaction. The fragment was reliably amplified with amounts of DNA (1–10 fg) that are less than that contained in a single *P. tabacina* sporangiospore. Use of this technique enabled the detection of *P. tabacina* DNA directly in local lesions, systemic vascular infections, and other infected parts of tobacco plants. The use of spore traps followed by amplification of this fragment facilitated the prediction of a local disease epidemic. Use of this highly specific and reliable detection method could prove valuable for regulatory agencies and for epidemiological and etiological studies.

Conventional methodologies for the detection of specific microorganisms include the use of selective culture media, differentiating spore traps, serology, and inoculation tests on different host species or cultivars. These approaches have not been satisfactory for the identification of organisms at extremely low propagule numbers and are cumbersome for use with obligate parasites (such as plant-pathogenic rust and mildew fungi), which cannot be cultured (6). Recently, highly sensitive and specific molecular markers have been developed and utilized for detection of animal pathogens (1,10) and a small number of plant pathogens (5,7). There remains a need for early and sensitive detection of obligate fungal parasites, which have traditionally been identified only after visual symptoms of disease are apparent.

Peronospora tabacina D. B. Adam, the oomycete that causes blue mold of tobacco, is an extremely destructive pathogen with aerially transported sporangiospores. This fungus belongs to the order Peronosporales, which includes the agents of downy mildew.

Since 1979, *P. tabacina* has caused billions of dollars of damage to tobacco worldwide (3). In North America, *P. tabacina* is endemic to areas of Texas, Mexico, and the Caribbean, where it infects year-round crops of commercial tobacco (*Nicotiana tabacum* L.) and wild species of tobacco such as *N. repanda* Willd. ex Lehm. Epidemics in cool areas, where the pathogen does not overwinter, depend on the extent and progress of the disease in the southern endemic regions as well as the weather and wind conditions that determine the movement of wind-blown sporangiospores into more northerly tobacco production areas of North America (2). As a result, northern epidemics are very difficult to predict or detect at an early stage.

Research was initiated to develop methodology for rapid and sensitive detection of *P. tabacina* infections and airborne spores for epidemiological and etiological studies. By examining polymerase chain reaction (PCR) fragments amplified with the use of short primers that promote random amplification of polymorphic DNA (RAPD) (18), we have isolated a fragment that can serve as a specific and sensitive probe for detection of *P. tabacina* sporangiospores. This finding has broad epidemiological and regulatory significance, and the approach may also be useful for detection of other agronomically and economically important fungal plant pathogens.

MATERIALS AND METHODS

Fungal isolates and DNA extraction. Isolates used in this study are listed in Table 1. *Pythium ultimum* Trow and *Phytophthora parasitica* Dastur var. *nicotianae* (Breda de Haan) Tucker were grown on potato-dextrose and oatmeal agar, respectively. All of the *Peronospora* isolates were grown in association with a host system (alfalfa for *P. trifoliorum* de Bary and tobacco for *P. tabacina*) as previously described (13). Extracted DNA was provided for *Phytophthora infestans* (Mont.) de Bary, *Peronosclerospora maydis* (Racib.) C. G. Shaw, and *Peronosclerospora sacchari* (T. Miyake) C. G. Shaw.

All DNA from the isolates collected in the United States was extracted at the University of Kentucky; DNA from foreign isolates was extracted under containment facilities at the USDA Foreign Disease-Weed Research Center, Ft. Detrick, Frederick, MD. Sporangiospores were collected from freshly sporulating lesions and filtered through a 3.0-μm pore size Millipore filtration system (Millipore, Bedford, MA) to remove plant and most other foreign matter. At least 1 mg of sporangiospores was crushed in liquid nitrogen by a mortar and pestle, and the resulting powder was treated as described by Panabières et al (12) unless otherwise stated.

PCR amplifications. RAPD employed 25-μl reactions containing four deoxynucleoside triphosphates (1.25 mM), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.0 mM $MgCl_2$, 0.01% gelatin, 1 μg of bovine serum albumin, 0.5 units of *Taq* DNA polymerase (AmpliTaq, U.S. Biochemical, Cleveland, OH), 5 ng of *P. tabacina* DNA, and 2 μM of the 10-base primer 5′-GGAGTACTGG-3′ (designated F-16 from Kit F, Operon Technologies, Alameda, CA). Temperature cycling parameters were 94 C for 60 s, 35 C for 60 s, and 72 C for 120 s in a model 480 thermocycler (Perkin-Elmer Cetus, Norwalk, CT). Afterward, 7 μl of each reaction was fractionated in a 1.25% agarose gel (SeaKem, HGT agarose, FMC, Rockland, ME). In all PCR experiments, separate reactions were included of a positive control with known DNA template to yield known product and a negative control with no DNA present to test for contamination of reagents.

After identification and sequence analysis, as described later, of a RAPD fragment designated 1602 from *P. tabacina* DNA (see Results), conditions were developed for specific amplification of the fragment. Two new primers were synthesized and designated 1602A and 1602B. The PCR mixture was as described above but with varying amounts of fungal DNA as described in the figure legends. After 4 min at 95 C, PCR employed 30 cycles of 95 C for 60 s, 62 C for 60 s, and 72 C for 60 s.

Cloning and characterization of fragments. Gel pieces were excised for extraction of DNA in preparation for cloning. Fragments were purified with the Geneclean II kit (Bio 101, La Jolla, CA) and ligated into pBluescript KS(+) plasmid vector (Stratagene Cloning Systems, La Jolla, CA). Clones that contained an insert were screened for hybridization to several genomic DNAs of the fungi described in Table 1. For each fungus, at least 1 μg of genomic DNA was transferred to Genescreen Plus nylon membrane (Dupont, Boston, MA) in a Hybri-Slot manifold (Bethesda Research Laboratories, Gaithersburg, MD). DNA was denatured in 0.25 N NaOH, 0.5 M NaCl, and 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), pH 7.0, and prehybridization was completed with 6× SSC, 50% formamide, 5× Denhardt's solution (0.5 g of Ficoll 400, 0.5 g of polyvinylpyrrolidone, and 0.5 g of bovine serum albumin), 0.1% sodium dodecyl sulfate, 0.1% $NaPO_4 \cdot 10H_2O$, 50 mM Tris-HCl, pH 7.5, and 50 μg of sheared single-stranded herring sperm DNA. Digoxigenin-labeled probes were generated from each clone by PCR with flanking vector primers (5′-GTAAAACGACGGCCAGT-3′ and 5′-ATTAACCCTCACTAAAG-3′), previously

TABLE 1. Oomycetes used in this study

Fungus	Isolate	Collection Year	Location	Source
Pythium ultimum	...	Unknown	Unknown	R. Ferriss[a]
Phytophthora				
parasitica var. *nicotianae*	Races 0 and 1	Unknown	Unknown	W. Nesmith
infestans	551	Unknown	Mexico	P. Tooley[b]
Peronosclerospora				
sacchari	23	Unknown	New Guinea	M. Bonde and G. Peterson[b]
maydis	...	Unknown	West Java	M. Bonde and G. Peterson
Peronospora				
trifoliorum	...	Unknown	Kansas	D. Stuteville[c]
tabacina	Ky79	1979	Kentucky	W. Nesmith
	Ky90 (3 isolates)	1990	Kentucky	M. Wiglesworth and W. Nesmith
	Tx92	1992	Texas	M. Wiglesworth
	Mx01, Mx04	1987	Mexico	M. Wiglesworth and C. E. Main[d]
	Pt33	1989	Mexico	M. Bonde and C. E. Main
	Bul	1988	Bulgaria	M. Bonde and C. E. Main
	Pt08	1987	France	M. Bonde and C. E. Main
	Pt11	1987	Hungary	M. Bonde and C. E. Main
	Pt28	1988	Guatemala	M. Bonde and C. E. Main
	GR63	1963	Germany	M. Bonde

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[b]Foreign Disease-Weed Science Research Center, Ft. Detrick, Frederick, MD.
[c]Department of Plant Pathology, Kansas State University, Manhattan.
[d]Department of Plant Pathology, North Carolina State University, Raleigh.

described buffers, and 0.25 nmol of digoxigenin-dUTP (8,9) plus 1.25 mM dATP, dGTP, and dCTP, and 1.0 mM dTTP. PCR was run for 30 cycles of 94 C for 1 min, 55 C for 1 min, and 72 C for 1 min. Hybridization was completed with a low-stringency wash of 2× SSC-0.1% sodium dodecyl sulfate at 53 C as described by Gebeyechu et al (4) with a DNA labeling and detection kit (Boehringer Mannheim, Indianapolis, IN). The single-stranded DNA form of the DNA clones was generated (14,16,17) and sequenced with the Sequenase kit (U.S. Biochemical).

Detection of single spores and systemic infections. Individual *P. tabacina* sporangiospores (isolate collected from Cumberland County, Kentucky, in 1992) were removed with a glass micromanipulator under a binocular microscope (Wild, Heerbrugg, Switzerland) and placed directly into 0.5-ml sterile microcentrifuge tubes containing 12.5 μl of sterile Milli-Q (Millipore) purified water. Samples were sonicated (50W Sonic Disrupter, model ASI, Tekmar, Cincinnati, OH) for 45 s at a setting of 70 and then centrifuged for 1 min at 15,000 *g*. One microliter from each tube was used as template for PCR reactions.

Systemically infected tobacco plants (*N. tabacum* cv. TN86 collected in 1992 from Monroe County, Kentucky, and *N. repanda* collected from Uvalde, Texas, in 1992) were rinsed in sterile Milli-Q water to remove external debris. A plug (approximately 1 mm^2) of vascular tissue was removed by scalpel, suspended in 500 μl of autoclaved Milli-Q water, and then sonicated as described above. Aliquots (1-μl) of the sonicated mixture were used as template for PCR. An uninfected plant was used as a negative control; a PCR tube with no DNA added was used as another negative control; and sporangiospore DNA of the Ky79 isolate of *P. tabacina* was used as the positive control.

Detection of aerial sporangiospores. Water agar plates (1%) were opened and placed overnight in several locations near and around the University of Kentucky, Lexington, and in Harrison County, Kentucky, during May 1992. Upon collection, microscopic examination was done to determine visually whether downy mildew-like spores were present. All material was collected from the plates by washing with 1 ml of Milli-Q water. Sonication and PCR reactions were as before. The positive control for this experiment was the aliquot from a plate with spores added. One negative control was the aliquot from an agar plate that was unexposed, and another negative control was a reaction with no template added.

Double-sided adhesive tape was placed on the front of an automobile that traveled in central Kentucky and from Hopkinsville to Princeton in western Kentucky. Acetone was used to remove the adhesive and collected material from the tapes. The suspension was dried under a vacuum, and the pellet was resuspended in 100 μl of autoclaved Milli-Q water and then sonicated as before. Aliquots (1-μl) of resuspended, sonicated material were used as template for PCR.

RESULTS

Three isolates of *P. tabacina* collected in 1990 from different locations in Kentucky (Table 1) had different RAPD profiles (Fig. 1). In order to establish whether profiles generated by the genomic DNA of *P. tabacina* were specific to *P. tabacina,* RAPD fragments were gel excised and cloned. Eight different fragments were cloned, and digoxigenin-labeled probes were generated from each. Slot blots of genomic DNAs from several different downy mildews and other Peronosporales were probed (Table 1). All clones showed homology to the fungi tested, with the exception of one cloned fragment, designated 1602 (Fig. 2), which specifically hybridized only to *P. tabacina* DNA.

The 232-bp sequence of fragment 1602 is shown in Figure 3. On the basis of this sequence, oligonucleotides 1602A and 1602B were synthesized (Fig. 3) for use in future PCR reactions. These produced a single PCR product of approximately 230 bp upon amplification from the RAPD products, and a PCR-generated digoxigenin-labeled probe was generated for hybridization to a

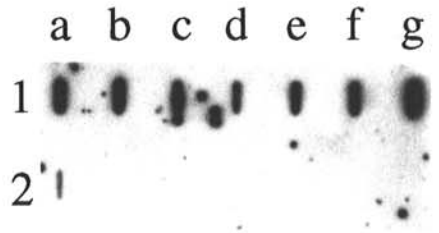

Fig. 2. Hybridization of fragment 1602 to DNA of *Peronospora tabacina* and other plant-pathogenic oomycetous fungi. DNA samples were from the following *P. tabacina* isolates: GR63, 1a; an isolate from Mexico collected in 1988, 1b; Pt33, 1c; Bul, 1d; Mx04, 1e; Pt11, 1f; Ky79, 1g; and Pt28, 2a. Other samples were from *Peronospora trifoliorum*, 2b; *Peronosclerospora sacchari* isolate 23, 2c; *Peronosclerospora maydis*, 2d; *Phytophthora parasitica* var. *nicotianae* race 1, 2e; and *Pythium ultimum*, 2f.

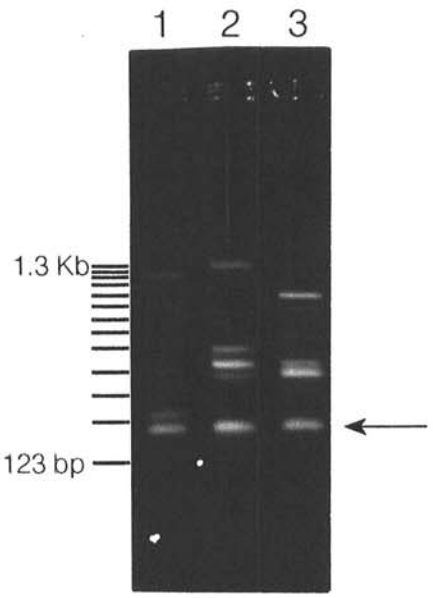

Fig. 1. Random amplified polymorphic DNA profile generated from several different isolates of *Peronospora tabacina.* The isolates were collected from infected burley tobacco, *Nicotiana tabacum,* at the University of Kentucky Spindletop Farm (lane 1), University of Kentucky Diagnostic Laboratory (lane 2), and Harrison County, Kentucky (lane 3), during the 1990 growing season. The arrow indicates the fragment derived from lane 2 that was cloned into 1602. Size standards are indicated.

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ggagtactggCTCTATGTACAGTGTCGGTAAGACTACCGAC
            1602A

ATATGTGATGTAGATACTATCGAATTGATGATATTCCTTTT

ATATGTGTCGTATTCGAAACATATTAATCGTGATCTTCCTA

TATTTTGATGACGGTGATATCGGCCGTTGCGTCTCCAAATG

TTCAGTCTTTGCTCGTCGAGCTTTCATATCCATAAAGAACG
                                    1602B

GTAGATGATCGACTGTccagtactcc
      1602B
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Fig. 3. Sequence of repetitive fragment 1602 from *Peronospora tabacina* DNA. The location of the random amplified polymorphic DNA primer (F-16) used in the original amplification is in lowercase and cannot be assumed to be completely homologous to the target sequence in the *P. tabacina* genome. Primers synthesized for polymerase chain reaction amplification of the fragment are underlined and named.

Southern blot of DNA from *P. tabacina* isolates Bul and Mx04. The Southern blot analysis indicated that there were many repeated copies of fragments showing homology to fragment 1602 in genomic *Eco*RI digests of both isolates (Fig. 4). The sequences homologous to 1602 were present on a series of fragments differing from one another by multiples of approximately 700 bp. In undigested DNA, homology was observed only in the large molecular size fraction of DNA.

When the oligonucleotides 1602A and 1602B were used in PCR, the 232-bp fragment was detected in all *P. tabacina* isolates but in no other species. When genomic DNA was used as PCR template, other fragments were amplified in addition to the 232-bp fragment (Fig. 5, lanes 3 and 4). Whether these are related to the repetitive series observed in Figure 4 is unknown, but they were not observed when DNA from any other species was used as template.

To determine the minimum amount of genomic DNA needed for reliable visible detection of fragment 1602 in an agarose gel, a dilution series of Pt33 DNA was used as template. The minimum level of visible product was amplified from between 100 fg and 1 pg (Fig. 6). Southern blot analysis was used to observe PCR products from reactions of 1–10 fg of DNA.

On the basis of these results, it was predicted that a single sporangiospore should contain enough target DNA to be detected by PCR. Sporulating lesions were collected from infected plants in Cumberland County, Kentucky, and sporangiospores were removed. Single sporangiospores were added directly to PCR buffer, sonicated for 45 s, and amplified. These experiments confirmed that the 232-bp fragment was detectable from a single sonicated sporangiospore (Fig. 5). In a total of 10 different single-sporangiospore amplifications, there were three amplification samples that did not amplify. In these tubes, there is the possibility that during sonication the contents of the sporangiospores, as well as the sporangiospores themselves, were propelled from the tubes. Other possibilities exist, but only with the use of single sporangiospores did there appear to be no product amplification when a known mixture of *P. tabacina* propagules was added.

The 232-bp fragment was also detected when single-lesion samples from infected leaves, seedlings with suspected systemic vascular infection (Fig. 5), and *N. repanda* leaves with suspected systemic infection (data not shown) were used. The criterion for determination that tobacco plants were systemically infected was based on visual symptoms of the affected plant (distortion, chlorosis, and stunting) and necrosis of the vascular tissues. In all cases, no amplifications were observed in the controls where either no sporangiospores were used or healthy plants were used.

On 15 June 1993, a series of water-agar plates was set out overnight in Harrison and Fayette Counties of Kentucky, where

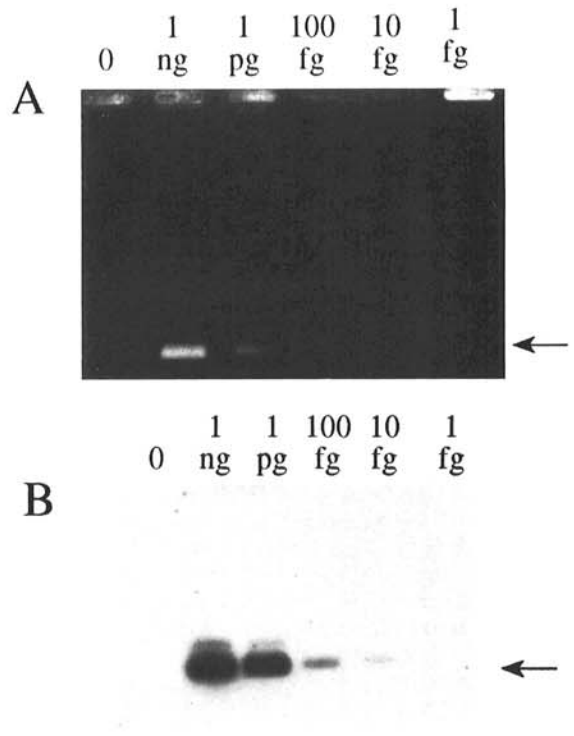

Fig. 5. Polymerase chain reaction (PCR) amplification of a fragment homologous to 1602 and Southern blot of various amounts of DNA ranging from 1 fg to 1 ng of *Peronospora tabacina* isolate Pt33. **A,** Ethidium bromide-stained 1.25% agarose gel of the PCR reactions; **B,** corresponding Southern blot hybridization. DNA concentrations of PCR template in both panels are noted, and the arrow indicates a 232-bp band.

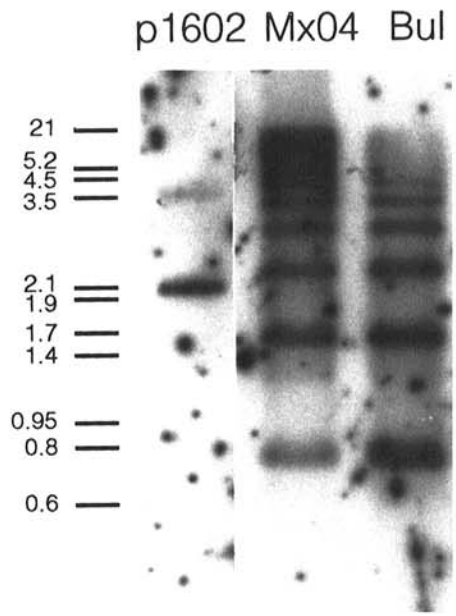

Fig. 4. Southern blot of *Eco*RI-digested DNA from *Peronospora tabacina* isolates Mx04 and Bul probed with digoxigenin-labeled fragment 1602. Lanes and sizes are as marked. Uncut fragment 1602 was used as the positive hybridization control.

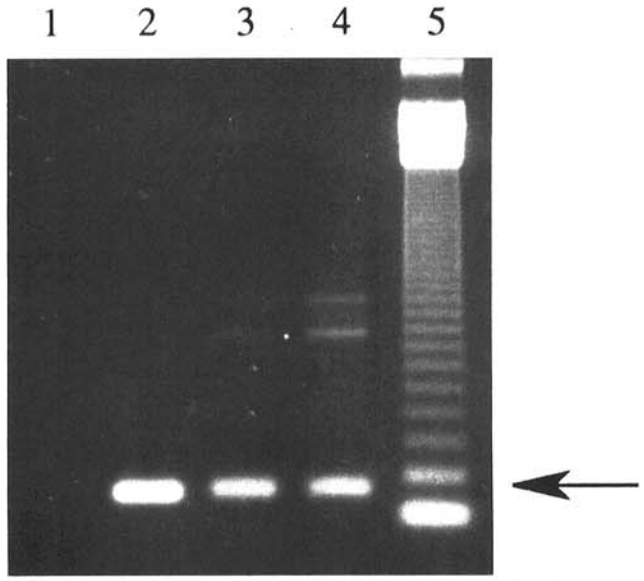

Fig. 6. Detection of DNA from single sporangiospores of *Peronospora tabacina* by polymerase chain reaction with oligonucleotide primers 1602A and 1602B. Lane 1, no DNA added; lane 2, 1602 used as template; lane 3, single spore of a *P. tabacina* isolate collected from Cumberland County, Kentucky, in 1992; lane 4, amplification of sonicated systemic vascular infections taken from tobacco samples collected in Monroe County, Kentucky; and lane 5, 123-bp ladder.

blue mold had not yet been detected but where field infections had been noted within 50 miles of the route taken (W. C. Nesmith, *unpublished*). Microscopic observation of these plates indicated that downy mildew and other non-downy mildew fungal spores were present. All spores present on these plates were sonicated and amplified for the presence of the fragment. In all cases, where downy mildew-like spores were visible, the 232-bp fragment was detected (Fig. 7). In instances where no downy mildew-like spores were present, no fragment was amplified.

A procedure in which double-sided adhesive tape was placed on an automobile driven along selected routes was also successful in detecting sporangiospores (Fig. 7). One route was taken from Hopkinsville to Princeton, Kentucky, on 29 May 1992 several days prior to the confirmation of field epidemics (W. C. Nesmith, *unpublished*). A second series of adhesive tape traps was used on a route from Cynthiana to Lexington, Kentucky, on 8 and 9 June 1992. On the date of the sampling, the blue mold pathogen had not been reported within 100 miles. However, 1 wk later, an epidemic of *P. tabacina* was detected in a tobacco field that was within 20 miles of the route previously taken.

DISCUSSION

The blue mold pathogen, like most obligate parasites, is an extremely difficult organism to identify before the onset of sporulation. The findings given here are the first to report a multicopy genomic fragment that enables the rapid detection of DNA present in single sporangiospores of *P. tabacina* or the mycelia in systemic vascular infections of tobacco, even in the presence of other contaminant organisms. The extension of this application for the detection of disease propagules before the occurrence of a field epidemic, for the study of the epidemiological and etiological nature of the blue mold pathogen, and for use in regulatory situations makes this detection assay valuable to the scientific and economic communities. In addition, methods for generation of this species-specific probe should be useful in the development of probes for other plant pathogens.

The use of RAPD markers for genetic studies is a recent development that enables detection and differentiation of individual strains and genotypes at the DNA level. However, a possible confounding aspect of the use the RAPD technique is the extreme sensitivity to contamination by other nontarget organisms. This is particularly important when obligate parasites are studied and in field surveys where contaminant or host DNA may be present. For this reason, RAPD fragments were cloned and used as probes to establish whether they were, in fact, homologous to the *P. tabacina* genome. This permitted the identification of the fragment described herein, which showed specific homology to *P. tabacina*. This sequence, designated 1602, was determined to be present as multicopies in both isolates of *P. tabacina* that were geographically and chronologically distinct. A number of *Eco*RI fragments with incrementally increasing sizes of 700 bp were homologous to the 1602 sequence. The repeated nature of the sequence undoubtedly enhanced the sensitivity of the PCR assay based on this fragment. However, the reason for the observed size series is unknown, but this pattern suggests that the cloned 232-bp fragment is part of a 700-bp tandem repeat unit.

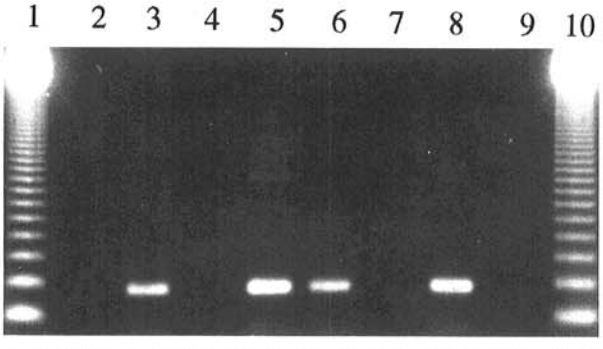

Fig. 7. Polymerase chain reaction amplification of fragment 1602 from various samples collected from agar spore traps, adhesive tape, and leaf infections. Lane 1, 123-bp ladder; lane 2, no DNA added; lane 3, *Peronospora tabacina* isolate Mx01 used as positive control; lanes 4 and 5, amplification from sonicated sporangiospores collected on adhesive tape; lanes 6 and 7, amplification from sonicated sporangiospores from open water agar plates; lane 8, *P. tabacina* isolate Ky79 used as positive control; lane 9, no DNA added; and lane 10, 123-bp ladder. Samples represented by lanes 4 and 7 had no downy mildew-like sporangiospores visible.

When oligonucleotide primers homologous to the ends of fragment 1602 were used, *P. tabacina* was detected by PCR at a very high level of sensitivity. The utility of the method depended on the minimal level of fungal material that could be reliably detected by the amplification of this fragment. The minimal visual detection of the amplified 232-bp fragment was between 100 fg and 1 pg of DNA. Since there have been determined to be 19–30 nuclei per sporangiospore of *P. tabacina* (15), the total amount of DNA within each sporangiospore may be as high as 1 pg. Because of the multiple copies of genomic DNA within a sporangiospore combined with the repetitive nature of the 232-bp fragment and the inherent sensitivity of PCR, the ability to detect *P. tabacina* by this method was extremely high. The amount of DNA required as template should be far less than that of a single sporangiospore. The sensitivity was further increased with Southern hybridization of the PCR-amplified product; *P. tabacina* was reliably detected with less than 10 fg of total DNA.

The practical potential of this technique was further established with sporangiospores and vascular infections, since these stages of the disease caused by *P. tabacina* cannot easily be distinguished. In both cases, the tissues were sonicated in order to disrupt the cellular contents. A concern in the use of sonication is extreme fragmentation of DNA. However, with the number of copies present in the genome and the small size of the fragment being amplified, it was assumed that sonication would result in release of a sufficient number of amplifiable sequence copies. This proved to be the case, although the use of sonication prior to amplification of single-copy or large fragments may not be recommended for other organisms.

The successful amplification of the 232-bp fragment from vascular infections of the blue mold pathogen has several immediate implications. First, it is noteworthy that the fragment reliably amplified in the presence of plant (and possibly bacterial) contaminants. Second, the presence of the 232-bp fragment in vascular tobacco material indicates that some aspect (e.g., mycelia or sporangiospores) of *P. tabacina* exists in these tissues. The only other technique that establishes the presence of *P. tabacina* in the vascular tissues is exhaustive electron and conventional microscopic studies. The PCR-based technique is far more sensitive and reliable and can be accomplished more easily and more quickly. Furthermore, use of this technique could detect the movement and development of *P. tabacina* within plant tissues, an aspect that is not well studied because of previous difficulty in detecting this fungus in planta.

Most downy mildews have airborne sporangiospores, some of which can be transported in the atmosphere over great distances. The sporangiospores of *P. tabacina* have the potential to travel hundreds of kilometers in the upper atmosphere (2,11). Thus, the availability of a technique to probe samples of air and establish the presence of the blue mold pathogen is important. At the collection site and route where positive identifications were made with water agar plates and adhesive tape, there were no known field epidemics of this pathogen in the vicinity until days or weeks later. However, advisories had been issued at the time because other conditions favored blue mold development. Although pathogen detection was associated with a later field epidemic, an important aspect for future studies is the ability to detect sporangiospores in the atmosphere. The ability to detect propagules in the atmosphere will facilitate research on tracking the blue mold fungus.

Currently, the only method for tracking the blue mold fungus is conducted by the North American Blue Mold Warning System (11), which is based on observed infections on commercial and wild species of tobacco. Hence, the time of arrival of sporangio-

spores is only estimated and confirmed retroactively on the basis of the development of disease symptoms. In many circumstances, this time delay is a crucial impediment to control, and more timely information would greatly facilitate implementation of control strategies.

Finally, the use of this molecular assay could prove valuable to world regulatory agencies. There is a great interest in world markets for tobacco leaf, transplants, and seed from the United States, but there is concern about the potential movement of *P. tabacina* with these materials. Therefore, disease-free seed or other clean tobacco materials are a necessity. The sensitivity of the PCR-based technique is valuable for the detection of possible infectious propagules and could prove valuable to national regulatory agencies to help prevent catastrophic disease spread.

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