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Detection and Quantification of *Peronospora tabacina* Using a Real-Time Polymerase Chain Reaction Assay

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Abstract

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Peronospora tabacina is an obligate plant pathogen that causes blue mold of tobacco. The disease is difficult to diagnose before the appearance of symptoms and can be easily spread in nonsymptomatic tobacco seedlings. We developed a real-time polymerase chain reaction (PCR) assay for *P. tabacina* that uses 5′ fluorogenic exonuclease (TaqMan) chemistry to detect and quantify pathogen DNA from diseased tissue. The primers and probe were designed using 5.8S ribosomal DNA sequences from 12 fungal and oomycete tobacco pathogens and 24 *Peronospora* spp. The PtabBM TaqMan assay was optimized and performed with a final concentration of 450 nM primers and 125 nM probe. The real-time TaqMan assay was assessed for sensitivity and the lower detection limit was 1 fg of DNA. The assay was specific for *P. tabacina*. None of the DNA from other tobacco pathogens, nonpathogens, or the host were amplified. The PtabBM TaqMan assay was useful for detection of *P. tabacina* in field samples, artificially inoculated leaves, roots, and systemically infected tobacco seedlings. The assay was used to quantify host resistance and it was possible to detect the pathogen 4 days postinoculation in both medium-resistant and susceptible tobacco cultivars. The real-time PCR assay for *P. tabacina* will be a valuable tool for the detection of the pathogen and of use to regulatory agencies interested in preventing the spread of blue mold.

Peronospora tabacina D.B. Adam is the causal agent of blue mold or downy mildew of tobacco (20,22). The pathogen is a fungus-like organism and is a member of the phylum Oomycota. *P. tabacina* is an obligate parasite, mostly restricted to the genus *Nicotiana*. Airborne sporangiospores of the pathogen can be transported over long distances into the United States to infect tobacco leaves from tobacco grown in the Caribbean basin and on weed hosts (23).

Since 1979, blue mold has become a devastating disease of tobacco fields each year and causes epidemics in seed beds, transplants, and production fields in humid and temperate regions of the southeastern and eastern United States, Canada, Central America, and countries bordering the Caribbean basin (2,7,8,18,23,27,35). Blue mold is also a problem in South America (16,21) and southwestern and southeastern Europe, the Middle East, and North Africa (9,26,30).

The pathogen can also be dispersed via infected transplants. In some cases, transplants that appear healthy may actually be infected. The latent period is 5 to 7 days after inoculation (28). Commercialization and sale of tobacco transplants from state to state is common and can increase the risk of disease spread and introduction of the pathogen into new areas.

In severe situations, *P. tabacina* may cause systemic infections. Systemic infection was first reported in Italy by Gigante (6,10) and in Australia by Mandryk (24,25). Factors affecting the development of systemic infections are not completely understood (27). Systemic symptoms of blue mold usually affect entire tobacco plantations, and can cause high economic losses. It is possible for the pathogen to remain latent until environmental conditions become favorable and plants finally express symptoms (6). Systemic infection results in leaf curl, chlorosis, stunting, and death of the terminal bud and the abnormal emission of bottom shoots. The presence of internal necrosis is diagnostic and affects the cambium and phloem tissues. Generally, there is no sporulation on the leaves, which makes identification very difficult (6,24,27).

Blue mold is fairly easy to diagnose after the symptoms and signs appear. The detection is typically based on symptomology and the presence of diagnostic clusters of sporangiospores on the underside of infected leaves (23,26). Detection of the pathogen in symptomless plants is critical for growers and extension agents. Because sporangiospores can spread rapidly from one field to another, chemical control application prior to sporulation is more effective than after sporulation has occurred. Molecular diagnostic tools can provide rapid and reliable detection for early disease development and increase confidence in the identification, even when the pathogen cannot be isolated (1,4,5,13). Ristaino *et al.* (29) and Tsay et al. (39) used the internal transcribed spacer (ITS) and 5.8S ribosomal DNA (rDNA) sequences of *P. tabacina* to develop specific polymerase chain reaction (PCR) primers for rapid detection of the pathogen. Wiglesworth et al. (42) used random amplified polymorphic DNA probes for the identification of the pathogen. PCR methods can be used directly on infected plant material, which is useful because *P. tabacina* is not culturable.

Real-time PCR has been used in studies for the detection of *Peronospora* spp., including *P. lamii* (3), *P. sparsa* (15), and *P. parasitica* (5), but has not been developed for *P. tabacina*. With the advent of quantitative real-time PCR (QPCR), it is possible to accurately quantify a specific pathogen within a host plant.

The objectives of this study were to (i) design, develop, and test specific PCR primers and a TaqMan probe for detection of *P. tabacina*; (ii) validate the real-time PCR assay using DNA isolated from pure cultures, fresh and frozen diseased tobacco leaf samples from the field, and artificially inoculated tobacco plant tissues; and (iii) establish a quantitative assay for *P. tabacina* detection.

Materials and Methods

***P. tabacina* isolates.** Seventy isolates of *P. tabacina* collected from 11 U.S. states and 11 countries and stored in cryogenic conditions were used in this study (Table 1). The collection is housed in

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Table 1. *Peronospora tabacina* isolates collected between 1945 and 2007 from U.S. and international sources and tested by real-time polymerase chain reaction

Isolate	Year	Source	Collector and location
United States samples			
CT991	1999	Connecticut	J. LaMondia, University of Massachusetts
CT021	2002	Connecticut	J. LaMondia, University of Massachusetts
FL002	2000	Florida	T. Kucharek, University of Florida
FL08	2008	Florida	A. Gevens, University of Florida
GA992	1999	Georgia	P. Bertrand, University of Georgia
GA996	1999	Georgia	A. Csinos, University of Georgia
GA995	1999	Georgia	A. Csinos, University of Georgia
GA08	2008	Georgia	J. Jacobs, University of Georgia
KY79	1979	Kentucky	B. Nesmith, University of Kentucky
KY206	2006	Kentucky	K. Seebold, University of Kentucky
KY062	2006	Kentucky	K. Seebold, University of Kentucky
KY071	2007	Kentucky	K. Seebold, University of Kentucky
BPT	1945	Texas	E. E. Clayton, Texas A&M University
Hadley	2004	Maryland	P. Shoemaker, North Carolina State University
BPTS	1945	Texas	E. E. Clayton, Texas A&M University
RPT83	1983	North Carolina	C. Main, North Carolina State University
Bertie	1983	North Carolina	R. Rufty, North Carolina State University
PT87W	1987	North Carolina	C. Main, North Carolina State University
Waynesville1	1998	North Carolina	J. Ristaino, North Carolina State University
NC998	1999	North Carolina	P. Shoemaker, North Carolina State University
NC9911	1999	North Carolina	P. Shoemaker, North Carolina State University
NC997	1999	North Carolina	T. Melton, North Carolina State University
NC994	1999	North Carolina	G. Pate, North Carolina State University
NC0212	2002	North Carolina	P. Shoemaker, North Carolina State University
AV041	2004	North Carolina	F. Bolick, North Carolina State University
BU041	2004	North Carolina	S. Holloway, North Carolina State University
BU061	2006	North Carolina	K. Ivors, North Carolina State University
PennB	1998	Pennsylvania	J. Yocum, Pennsylvania State University
Colum	1998	Pennsylvania	J. Yocum, Pennsylvania State University
PE05	2005	Pennsylvania	B. Maksymowicz, University of Kentucky
PE07	2007	Pennsylvania	B. Maksymowicz, University of Kentucky
PE08	2008	Pennsylvania	B. Green, Pennsylvania
BPTP	1945	Texas	E. E. Clayton, Texas A&M University
TX84	1984	Texas	B. Nesmith, University of Kentucky
TX062	2006	Texas	M. Black, Texas A&M University
Mosel	1998	Virginia	J. Ristaino, North Carolina State University
VA011	2001	Virginia	Avery, Virginia Tech
VA08	2008	Virginia	R. Wells, Virginia Tech
OH-AD-04	2004	Ohio	R. Stevenson, Ohio State University
OH-CL-04	2004	Ohio	R. Stevenson, Ohio State University
OH-HL-04	2004	Ohio	R. Stevenson, Ohio State University
OH-GA-04	2004	Ohio	R. Stevenson, Ohio State University
OH-JK-04	2004	Ohio	R. Stevenson, Ohio State University
OH-BR-06	2006	Ohio	R. Stevenson, Ohio State University
OH-CL-06	2006	Ohio	R. Stevenson, Ohio State University
OH-GA-06	2006	Ohio	R. Stevenson, Ohio State University
International samples			
123046	1988	Bulgaria	G. L. Peterson, USDA, Ft. Detrick, MD[a]
DR022	2002	Dominican Rep.	I. Abreu, FERQUIDO
DR051	2005	Dominican Rep.	A. Ubiera, FERQUIDO
DR054	2005	Dominican Rep.	A. Ubiera, FERQUIDO
DR062	2006	Dominican Rep.	M. Peralta, Tabadom Holding Inc.
DR064	2006	Dominican Rep.	M. Peralta, Tabadom Holding Inc.
DR066	2006	Dominican Rep.	M. Peralta, Tabadom Holding Inc.
123392	1989	Guatemala	G. L. Peterson, USDA, Ft. Detrick, MD[a]
123364	1987	France	R. Delon, Institut du Tabac-Altadis
FR184	2005	France	J. L. Verrier, Institut du Tabac-Altadis
FR194	2005	France	J. L. Verrier, Institut du Tabac-Altadis
FR178	2005	France	J. L. Verrier, Institut du Tabac-Altadis
FRy1	1980s	France	J. L. Verrier, Institut du Tabac-Altadis
123350	1963	Germany	G. L. Peterson, USDA, Ft. Detrick, MD[a]
123368	1960	Hungary	G. L. Peterson, USDA, Ft. Detrick, MD[a]
123340	1963	Lebanon	G. L. Peterson, USDA, Ft. Detrick, MD[a]
NIC063	2005	Nicaragua	N. Placencia, Segovia Cigars
NIC065	2005	Nicaragua	N. Placencia, Segovia Cigars
123347	1989	Mexico	P. Shoemaker, North Carolina State University
123296	1989	Mexico	P. Shoemaker, North Carolina State University
Mex	1999	Mexico	R. Rufty, North Carolina State University
MX02	2002	Mexico	V. Nikolaeva, Amapa, Nayarit
123349	1989	Poland	G. L. Peterson, USDA, Ft. Detrick, MD[a]
123344	1989	Poland	G. L. Peterson, USDA, Ft. Detrick, MD[a]

[a] Samples belong to a collection formerly located at the United States Department of Agriculture (USDA), Ft. Detrick, MD and moved to the Department of Plant Pathology at North Carolina State University in 2004.

the Department of Plant Pathology at North Carolina (NC) State University and is located in a BS Level 2 containment laboratory (34). Tobacco leaves with lesions were collected by collaborators, placed inside plastic bags with a wet towels to retain humidity and maintain sporulation, and sent to NC State University. International samples were sent using a kit mailed to collaborators that contained Eppendorf tubes with dimethyl sulfoxide (DMSO, 10% solution), labels, plastic pipettes, gloves, and ice packets. In the laboratory, the new isolates of *P. tabacina* were inoculated on tobacco seedlings to increase the amount of sporangia for subsequent cryostorage. Inoculation was performed on one of two susceptible cultivars of either *Nicotiana repanda* or *N. tabacum* cv. *xanthi*. Sporangiospores were collected from the leaves and stored at –4°C on 10% DMSO for 1 day and later transferred to liquid nitrogen at –210°C.

Other foliar and soilborne fungal pathogens of tobacco were grown on potato dextrose agar (PDA; Table 2). Mycelia from cultures in petri dishes were transferred to potato dextrose broth, grown at 25°C for 3 to 5 days, and then filtered to collect mycelia. Roughly 100 mg of mycelia were placed in a sterile 1.5-ml microcentrifuge tube for DNA extraction as described below and stored at –20°C for future use.

DNA extraction. DNA was extracted using the hexadecyltrimethylammonium bromide (CTAB) procedure with some modifications in all of the experiments (38,41). DNA was extracted from sporangiospores, fresh or dried tobacco leaf lesions (10 mg of tissue), or sporangiospores washed from fresh lesions (by vortexing 10 mg of tissue in water and removing tissue). If sporangiospores were no longer viable and inoculation on the tobacco plants was not possible, DNA extraction was performed directly from the cryogenic spore solution. Tissue was placed in sterile 1.5-ml microcentrifuge tubes containing 0.2 g of glass beads to which 150 µl of extraction buffer (0.35 M sorbitol, 0.1 M Tris, 0.005 M EDTA

Table 2. Isolates of *Peronospora tabacina,* other tobacco pathogens, and related Oomycetes used to develop primers and probe and test the specificity of the real-time polymerase chain reaction assay

Species	GenBank accession no.[a]	Source[b]	Host	Collector[c]
Tobacco pathogens				
Alternaria alternata[d]	DQ059568, DQ323699, AY751456	North Carolina	*Nicotiana tabacum*	H. Spurr
Cercospora nicotianae[d]	DQ059569, DQ059571	North Carolina	*N. tabacum*	M. Daub
Peronospora tabacina[d]	DQ067896, DQ067897, DQ067898, DQ067899, DQ067900, DQ665672, AY198289	North Carolina, GenBank	*N. tabacum*	J. Ristaino, M. Thines, and H. Voglmayr
Phytophthora glovera[d]	DQ059570, AF279126, AF279127, AF279128	Brazil	*N. tabacum*	D. Shew
Phytophthora parasitica[d]	DQ059571	North Carolina	*N. tabacum*	D. Shew
Pythium aphanidermatum[d]	DQ059572, DQ298521, DQ298523, AF271227, AB160845, AF271227	North Carolina	*N. tabacum*	J. Ristaino
Pythium dissotocum[d]	DQ059573, AF271228, AF330184	North Carolina	*N. tabacum*	D. Shew
Pythium myriotylum[d]	DQ059574, DQ102701, DQ222438	North Carolina	*N. tabacum*	W. Gutierrez
Phythium ultimum[d]	DQ059575, AF271225, DQ211527, AY986952	North Carolina	*N. tabacum*	W. Gutierrez
Rhizoctonia solani[d]	DQ059576	North Carolina	*N. tabacum*	J. Ristaino
Sclerotinia sclerotiorum[d]	DQ059577	North Carolina	*N. tabacum*	D. Shew
Sclerotium rolfsii[d]	DQ059578	North Carolina	*N. tabacum*	J. Ristaino
Thielaviopsis basicola[d]	DQ059579	North Carolina	*N. tabacum*	D. Shew
Other Oomycete spp.				
Hyaloperonospora parasitica[d]	AY210988.1	Virginia	*Arabidopsis thaliana*	J. McDowell
Hyaloperonospora niessleana	AY531465.1	GenBank	*Alliaria petiolata*	Goeker et al.
Peronospora arabis-alpinae	AY531466.1	GenBank	*Brassicaceae*	Goeker et al.
Peronospora arborescens	DQ885384.1	GenBank	*Papaver alpinum*	Landa et al.
Peronospora arenariae	AY198280	GenBank	*Moehringia trinervia*	H. Voglmayr
Peronospora arthurii	AY198284	GenBank	*Oenothera biennis agg.*	H. Voglmayr
Peronospora astragalina	AY608608.1	GenBank	*Astragalus membranaceus*	Choi et al.
Peronospora boni-henrici	AY198286	GenBank	*Chenopodium bonus-henricus* L.	H. Voglmayr
Peronospora campestris	AY608609.1	GenBank	*Amaranthaceae*	Choi et al.
Peronospora chenopodii-polyspermi	AY198291	GenBank	*Chenopodiaceae*	H. Voglmayr
Peronospora claytoniae	AY198281	GenBank	*Minerlettuce*	H. Voglmayr
Peronospora corydalis	AY211015.1	GenBank	*Fumariaceae*	Choi et al.
Peronospora cristata	DQ885375.1	GenBank	*Papaver somniferum*	Landa et al.
Peronospora destructor	DQ885385.1	GenBank	*Allium* spp.	Landa et al.
Peronospora erysimi	AY531461.1	GenBank	*Erysimum* herbaceous plants	Goeker et al.
Peronospora farinosa	AF528558	GenBank	*Beta* spp.	Choi et al.
Peronospora holostei	AY198283	GenBank	Chickweed	H. Voglmayr
Peronospora iberidis	AY531461.1	GenBank	Brassicales	Goeker et al.
Peronospora lepidii-sativi	AY531463.1	GenBank	Pepperweed	Goeker et al.
Peronospora manshurica	AY211019.1	GenBank	*Glycine max*	Choi et al.
Peronospora polygoni	AY198280	GenBank	*Polygonum aviculare*	H. Voglmayr
Peronospora sparsa	AY608610.1	GenBank	*Prunus laurocerasus*	Choi et al.
Phytophthora citricola	AM235209	GenBank	Citrus	Zea-Bonilla et al.
Phytophthora infestans[d]	EF173472	Ecuador	*Solanum tuberosum*	J. Ristaino
Phytophthora ramorum[d]	DQ873514	Georgia	*Lithocarpus densiflora*, *Quercus* spp.	J. Ristaino
Pseudoperonospora cubensis[d]	EF050035.1	North Carolina	Cucurbits	G. Holmes
Pseudoperonospora humuli[d]	EF126356.1	Oregon	*Humulus lupulus*	D. Gent

[a] GenBank accession numbers of sequences used to design primers and probe for the TaqMan assay.

[b] Source indicates state and country where the samples were collected or GenBank when the accession numbers were used.

[c] Indicates the name of sample collector or the first author for the accession numbers from GenBank. Harvey Spurr, North Carolina State University (NCSU); Margaret Daub, NCSU; David Shew, NCSU; Jean Ristaino, NCSU; Walter Gutierrez, NCSU; John Yocum, Pennsylvania State University; John McDowell, Virginia Tech; Gerald Holmes, Valent Chemical Co.; David Gent, Oregon State University.

[d] Species used to test the *P. tabacina* TaqMan assay.

[pH 7.5], and 0.02 M sodium bisulfite) was added and each sample was macerated using a Konte pestle attached to a drill. Nuclei lysis buffer (150 µl; 0.2 M Tris, 0.05 M EDTA [pH 7.5], 2.0 M NaCl, 2% CTAB, and 60 µl of 5% sarkosyl [N-laurylsarcosine]) was added and tubes were vortexed and incubated at 65°C for 15 to 30 min. After incubation, one volume (300 µl) of chloroform/isoamyl alcohol (24:1) was added to each tube and tubes were centrifuged for 15 min at 13,000 × *g* at room temperature. The aqueous phase was removed to a new tube and chloroform extraction was repeated. DNA was precipitated overnight at –20°C in 0.1 volume of 3 M sodium acetate (pH 8.0) and 2 volumes of cold 100% ethanol. The supernatant was discarded; pellets were washed with 70% ethanol and dried at room temperature. DNA was suspended in Tris-EDTA (TE; 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). A 1:10 dilution of DNA was used.

Growth of tobacco plants. *N. repanda* seed were planted in soil consisting of one-third peat-lite and two-thirds gravel, covered lightly with sand, and misted with a nutrient solution in a growth chamber at 25°C under a photoperiod of 12 h of light and 12 h of darkness. Seedlings at the two-leaf stage were moved to the greenhouse at 25°C and natural light conditions until inoculation. For inoculation, the tobacco seedlings were moved to growth chambers and placed in separate plastic boxes to prevent cross contamination among isolates. In some experiments, detached leaves were inoculated after placing inside inverted petri dishes with 30% water agar to maintain 95% humidity.

Inoculation of tobacco with *P. tabacina.* Isolates of *P. tabacina* were thawed at room temperature and washed four times with 1 ml of autoclaved distilled water by centrifugation at 12,000 rpm to eliminate the DMSO residue. Plants were moved from the greenhouse to a growth chamber set at 22°C with a 10-h photoperiod of light, then a 13-h photoperiod of darkness at 18°C that was interrupted in the middle with a 1-h light period. Light was provided by a combination of incandescent and fluorescent lights at an intensity of 200 µmol/m^2/s (34). Plants were inoculated by either spraying a solution of 10^8 sporangia/ml directly on the underside of the tobacco leaf, transferring washed sporangiospores onto the leaf surface using a cotton swab, or by pipetting one 10-µl drop of sporangiospores onto the leaf. After 10 to 12 days, signs of sporulation were observed and the leaf lesions were collected to perform the DNA extraction and PCR reactions.

Primer and probe design. Primers and a specific probe to identify *P. tabacina* were designed using the amplicon generated by primers ITS4 and ITS5 (42) of the rDNA cluster and Primer Express 1.5 software (Applied Biosystems, Foster City, CA). GenBank accession numbers are shown in Table 2. The following criteria for the probe design were used: melting temperature 8 to 10°C higher than the melting temperature of the primers, 15 to 30 bp in length, and the total number of Gs or Cs in the last five nucleotides at the 3′ end not exceeding two. The mismatching nucleotides were positioned as close as possible to the middle of the probe rather than at the ends while avoiding positions with secondary structures (13).

In total, 37 sequences were used from tobacco pathogens, including 7 sequences of *P. tabacina*, from the rDNA region located between primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (Table 2). Eighteen sequences of other tobacco pathogens and six of *P. tabacina* were reported by Ristaino et al. (29) in a previous study. An additional 27 sequences from other Oomycetes spp. were obtained from the GenBank database at the National Center of Biotechnology Information (Table 2) and compared using the BLASTn database to look for variable regions useful for primer and probe development. In all, 64 sequences of genera *Phytophthora*, *Pythium*, *Peronospora*, *Hyaloperonospora*, and *Pseudoperonospora* were aligned using BioEdit Sequence Alignment Editor version 7.0.5.3 (Table 2).

The primers and probe were designed inside the 5.8 rDNA (Fig. 1). The probe was labeled with the 6-carboxyfluorescein fluorescent dye (6-FAM) and the minor groove binder (MGB; 19). The amplicon generated by primers Ptab2F (5′-GCTGCGAACTGC GATACG-3′), and Ptab2R (5′-CCGAAAGTGCAATATGCGTTC AAAA-3′) and the PtabBM (5′-CTGAATTCCGCAATTCGT-3′) probe consists of a fragment of 86 bases. All reactions were performed in 0.2 ml of optical grade plates (Applied Biosystems) in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

Real-time PCR reactions were performed in a total reaction volume of 20 µl containing 1 µl of genomic DNA at a concentration of 10 ng/µl in specific tests (determined with a NanoDrop 1000 spectrophotometer; Thermo Fisher Scientific), 10 µl of 2× TaqMan Universal Master Mix (Applied Biosystems), 1 µl of the 20× Assay Mix (Applied Biosystems) with the two primers Ptab2F and Ptab2R (18 µM) and the probe PtabBM (5 µM), and 8 µl of RNase-free water (Fisher Scientific, Pittsburgh). The thermal cycling parameters were 50°C for 2 min and 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The threshold line or level of detection was set at fluorescence (ΔRn) of 0.2. A cycle

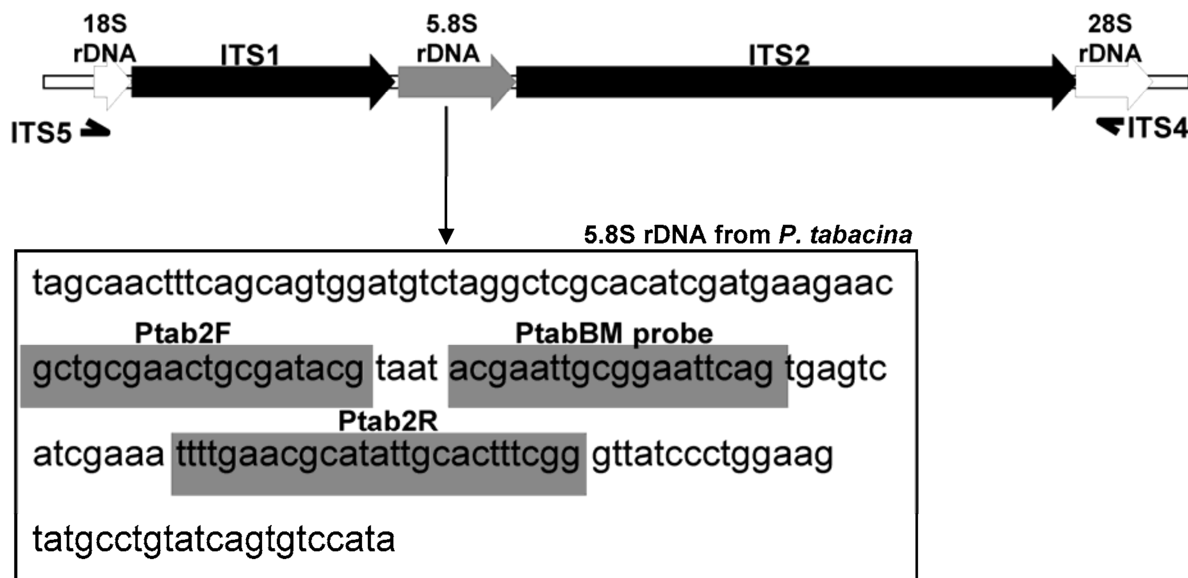

Fig. 1. Diagram of the internal transcribed spacer (ITS) regions and 5.8S ribosomal DNA (rDNA) of *Peronospora tabacina* indicating the location of designed primers and probe for the PtabBM TaqMan assay.

threshold (Ct) was scored positive between 16 and 35 and negative above 35.

An additional universal primer set and TaqMan probe included with the ABI real-time kit containing eukaryotic 18S rRNA Endogenous Control, labeled as VIC dye-MGB, was used to control for successful DNA extraction (Applied Biosystems). The primer produces an amplicon of 187 bases. The 2× TaqMan Gene Expression Master Mix (Applied Biosystems) was used to improve the amplification of both targets in a multiplex real-time PCR reaction of the more abundant target detected by the universal primer and the less abundant target detected by the Ptab primers and probe.

To perform the PtabBM TaqMan assay for quantification of *P. tabacina*, the multiplex reactions were performed on separate wells on the plate. One well was used to test the PtabBM primer/probe and another well with the same DNA sample was used for the universal primer/probe.

Each series of amplifications using the TaqMan assay included a duplicate of each sample and two no-template negative controls to test for contamination, DNase- and RNase-free water, as well as DNA from a healthy tobacco leaf. Because the negative controls are used to assess contamination, they were assessed at a higher Ct than the positive controls.

It was important to determine whether the detection of DNA from the pathogen could be affected by cross contamination. To determine whether the presence of tobacco DNA and coextracted host compounds affected the amplification of target DNA, DNA extracted from inoculated tobacco leaves with *P. tabacina* was compared with DNA extracted from pure sporangiospores. The amount of extracted plant DNA used in this experiment was 10 mg, the same as when field plants were sampled.

Analysis of the data. Sequence Detection Software System (version 1.2.3; Applied Biosystems) was used to analyze the data. This software works directly with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The software was used to define plate standards and negative and unknown values for the samples, control thermal cycler reaction settings, generate amplification plots of Ct values and Rn values (measure of reporter signal), generate standard curves, determine final Ct values, and quantify sample concentrations and standard deviations of the mean Ct values for all reactions.

Optimization of the PtabBM TaqMan assay. The concentrations of the primers and probe were optimized. The primer and probe concentrations recommended by Applied Biosystems of 900 nM primers and 250 nM probe were used and then three dilutions of primer and probe were tested, including 450, 250, and 125 nM primers and 125, 62.5, and 31.2 nM probe. Six isolates of *P. tabacina* (PennB, CT991, FR184, 123347, PE05, and GA995; Table 1) and a non-template water control were tested in duplicate on a plate and the experiment was repeated twice. The detection Ct value and the estimated DNA concentration were analyzed as well as the quality of the curve for each of the reactions tested for this assay.

Sensitivity of the PtabBM TaqMan assay. To calculate amplification sensitivity of the assay, a 10-fold serial dilution of DNA was quantified and tested, using concentrations of 450 nM primers and 125 nM probe. A 10-fold serial dilution of DNA extracted from sporangiospores was run initially and in each of the real-time PCR assays conducted, in order to compare DNA concentrations of known and unknown samples and calculate DNA concentrations. Three different isolates of *P. tabacina* were used for the dilutions (PennB, BU061, and Waynesville1). The DNA concentration used in the reaction was determined with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The serial dilution of DNA consisted of an initial concentration of 10 ng/µl diluted in a series of 1:10 from 10 ng/µl to 10 ag/µl (10 ng/µl, 1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl, 100 fg/µl, 10 fg/µl, 1 fg/µl, 100 ag/µl, and 10 ag/µl). A non-template negative control and DNA from a healthy tobacco leaf were included. All dilutions were done in duplicate on the plate and the experiment was repeated twice. There were 120 total reactions (10-fold dilution × three isolates × two replications × two experiments).

The results of the real-time PCR reactions were analyzed by plotting the log of template concentration of DNA against Ct values. PCR efficiency was calculated with the formula $E = (10^{(-1/\text{slope})} - 1) \times 100$, where E is the amplification efficiency and the slope is derived from the plot of log of template concentration versus Ct (17). A slope of 3.32 translates into 100% efficiency of amplification.

Conventional PCR assay. Seventy isolates of *P. tabacina* (Table 1) used to test the TaqMan assay were also tested using the conventional PCR with primer PTAB and the ITS4 primers reported previously (29). Each PCR reaction consisted of the following: 1 µl of genomic DNA (1:100 dilution of original DNA extract in TE buffer, about 10 ng/µl) was added to a 49-µl master reaction mixture containing 5 µl of 10× PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, and 500 mM KCl, pH 8.3), 35.25 µl of sterile distilled H_2O, 1.8 µl of 10 mM $MgCl_2$, 2 µl of 2.50 mM dNTPs, 2 µl of 10 µM PTAB primer (5′-ATCTTTTTGCTGGCTGGCTA-3′), 2 µl of 10 µM ITS4 primer (5′-TCCTCCGCTTATTGATATGC-3′), 0.25 µl of bovine serum albumin (Roche, Nutley, NJ), and 0.2 µl of Taq polymerase (Invitrogen, Carlsbad, CA). Thermal cycling parameters were initial denaturation at 96°C for 2 min, followed by 35 cycles consisting of denaturation at 96°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. Thermal cycling was followed by a final extension step at 72°C for 10 min. Amplified products were separated on 1.6% agarose gels containing ethidium bromide at 0.5 µg/ml with 1× Tris-borate-EDTA running buffer. DNA bands were visualized using UV light on a transluminator (Bio-Rad, Hercules, CA.).

Specificity of the PtabBM TaqMan assay. The specificity of the TaqMan assay was tested using DNA from 6 isolates of *P. tabacina* (WI-07, OH-AD-04, OH-DL-04, PennB, BU061, and Waynesville1; Table 1); DNA from 12 different tobacco pathogens, and DNA from 5 other Oomycete spp.; including *Pseudoperonospora humuli*, *Hyaloperonospora parasitica*, *P. cubensis*, *Phytophthora infestans*, and *P. ramorum* (Table 2). DNA from a noninoculated tobacco leaf and water were used as negative controls. Primer and probe concentrations were 450 and 125 nM, respectively, and the 10-fold dilution using *Peronospora tabacina* isolate PennB was used to compare DNA concentration of the samples tested. In total, 70 real-time reactions were performed (23 isolates, 10 serial dilutions, and two controls × two replications). All the reactions were performed in duplicate and the experiments were done twice.

DNA extracted from the isolates of *P. tabacina* (Table 1) was tested using the TaqMan assay. A multiplex reaction was used with concentrations of 450 nM primers and 125 nM probe. A 10-fold dilution of *P. tabacina* (BU061) was used to determine DNA concentrations of the samples tested. All reactions were performed in duplicate and the experiment was preformed twice. In total, 170 real-time reactions were performed for the detection of the pathogen.

Detection of *P. tabacina* in infected field samples, artificially inoculated leaves, seedlings and roots. The NaOH/Tris DNA extraction method was compared with the CTAB DNA extraction method to test the quality and reliability of both methods on extraction of *P. tabacina* from infected tissue (40). For this experiment, three infected leaf samples (OH-AD-04, OH-CL-04, and WI-07), a chlorotic lesion, a sporulating lesion, and a solution of sporangiophores were used and DNA was extracted. For the NaOH/Tris method, a small amount of sporangiospores or leaf tissue was placed in a 1.5-ml microcentrifuge tube. A volume of 90 µl of 0.5 M NaOH was added and maceration was performed with a clean Konte pestle until the samples were liquefied. A total volume of 3 µl of the solution was transferred immediately to a new tube and 300 µl of 100 mM Tris buffer, pH 8.0, was added (40). The tube was vortexed and placed on ice. The samples were used directly on the PCR reaction or stored at –20°C.

Field samples of tobacco leaves with signs and symptoms of blue mold were collected. Real-time PCR was done with samples from two fields in 2007 (two samples per field) and four fields in

2008 (three samples per field) using fresh leaf lesions (Table 1). For each sample, CTAB DNA extraction was performed on 10 mg of tissue from each of three areas: the lesion area, an area 1 cm away from the lesion area, and 4 to 5 cm away from the lesion for each of the samples. In addition, a 10-fold dilution of DNA extracted from sporangiospores of the PennB isolate of *P. tabacina* was used to quantify DNA concentration in the lesions and a nontemplate water and healthy tobacco leaf control were used in all experiments. All of the reactions were performed in duplicate.

To determine whether the real-time PCR assay could be used for detection of the pathogen in leaves with nonsporulating chlorotic lesions, 10 detached leaves were artificially inoculated with the isolate WI-07. *N. repanda* leaves were inoculated with 1×10^8 spores/ml using a cotton swab and incubated in petri dishes for 6 days. When chlorotic nonsporulating lesions were observed, 10 mg of tissue was used for CTAB DNA extraction and the real-time assay was performed for each of the samples.

Late-stage lesions with visible sporulation (sporangia), chlorosis, and necrosis were sampled. In all, 25 seedlings growing under greenhouse conditions and 25 in vitro cultured *N. repanda* seedlings were artificially inoculated and stored in plastic boxes or in tissue culture containers, respectively. After 12 days, 10 mg of tissue from the sporulating lesions was removed and DNA was extracted with the CTAB method, and the real-time assay was performed for each of the samples as described previously.

Lesions from air-dried leaves from Ohio fields sent in 2004 that were stored in the freezer at –20°C were tested. Ten visible lesions (2 mg each) from different leaf samples (OH-AD-04, OH-CL-04, OH-HL-04, OH-GA-04, OH-JK-04, OH-BR-06, OH-CL-06, OH-GA-06, OH-JK-06, and OH-LA-06; Table 1) were tested and CTAB DNA extraction and real-time PCR was performed as described previously.

In total, 188 real-time PCR reactions were performed in the test on chlorotic, sporulating, and air-dried tobacco leaves. The assay used concentrations of 450 nM primers and 125 nM probe and a 10-fold dilution with *P. tabacina* isolate PennB to compare DNA concentration of the samples tested. Each single reaction was performed in duplicate. Controls included no-template samples and healthy tobacco leaf tissue. All experiments were done twice.

Tobacco plants were inoculated to induced systemic infection by injection with a sterile needle of solution of *P. tabacina* at 1×10^8 spores/ml into the stem of 10 susceptible *N. .repanda* plants. After 20 days with no sporulation on the leaves, tissue was collected from the leaves (0.1 g), stems (0.15 g), and roots (0.01 g) of each plant and CTAB DNA extraction and real-time PCR were performed as described previously. Each single reaction was performed in duplicate.

Twelve tobacco plants were inoculated and left for 30 days under 95% relative humidity, until the aerial part of the plant was consumed by the pathogen. Roots systems from each plant were removed from the soil and washed with distilled water; then, DNA was extracted and real-time PCR was performed on 0.01 g of root tissue.

PtabBM TaqMan assay to quantify host resistance. To quantify the colonization of pathogen and DNA concentration in tobacco leaf tissue during early stages of infection, the ratio of pathogen DNA to host DNA was determined. The PtabBM TaqMan assay was tested on eight tobacco genotypes that differ in resistance to blue mold. Samples included burley 'NC2002' and 'NC2000' and flue-cured 'Chemical Mutant' (resistance obtained after treating seed of flue-cured tobacco 'Virginia Gold' with triethylene iminotriazine), which are moderately resistant; burley 'TN90', which is considered tolerant; 'Xanthy' and flue-cured 'Hicks' and 'NC55', which are susceptible; and burley 'KY14', which is highly susceptible (23). Tobacco cultivars were provided by Dr. Ramsey Lewis from the Crop Science Department at North Carolina State University. Tobacco discs (wet weight 0.1 g, 2 cm in diameter) were inoculated using a cotton swab with a solution of isolate VA-08 (Virginia 2008) of *P. tabacina* at 1×10^8 sporangia/ml as previously described. Three discs were placed in separated, inverted petri dishes with 30% water agar. The discs were inoculated and sampled 0, 2, 4, 6, 8, and 10 days after inoculation and, in all, 144 CTAB DNA extractions were performed from each leaf disk and cultivar from each sample time. Two noninoculated control leaves for each tobacco cultivar, a no-template control, and two noninoculated negative controls of *N. repanda* were also used.

To compare the most susceptible and resistant cultivars, DNA was extracted from the three discs of Chemical Mutant (36 samples) and KY14 (36 samples) and from the negative controls. The PtabBM TaqMan assay was used to detect the degree of the pathogen colonization over time. For each round of real-time PtabBM, TaqMan detection was performed using the universal primer in a different reaction plate, in order to ensure reporting by both fluorogenic probes and no interference on the quantification of the DNA. A concentration of 900 nM primers and 250 nM probe was used and a 10-fold dilution with PennB was used to compare DNA concentration of the samples tested. Each reaction was performed in duplicate and experiments were done two times. DNA concentrations of *P. tabacina* in Chemical Mutant and KY14 were compared between each treatment and between different sample times by analysis of variance using the Proc GLM procedure of SAS 9.1 (SAS Institute, Inc., Cary, NC); α levels < 0.05 were used to denote statistical significance.

Results

Primers and probe design. A real-time PCR assay for *P. tabacina* that uses 5′ fluorogenic exonuclease (TaqMan) chemistry was developed that detects and quantifies pathogen DNA from diseased tissue. The amplicon generated by the two primers Ptab2F and Ptab2R and the PtabBM probe consists of a fragment of 78 bases (Fig. 1).

The PtabBM probe had four mismatches when compared with the 27 sequences from *Peronospora* spp. and Oomycetes from GenBank (Table 2). The Ptab2F and Ptab2R primer sequences were 100% identical to many Oomycete pathogens, except for *Alternaria*, *Cercospora*, *Thielaviopsis*, *Sclerotium*, and *Sclerotinia* spp. (see supplementary figure). This suggests the interpretation that the specificity is located in the PtabBM probe.

The addition of universal primers and probe interfered with the quantification of the DNA from *P. tabacina* but not with the detection of the pathogen. Lower levels of DNA were detected from the same sample when the universal primers and probe were used in a multiplex reaction. For example, when *P. tabacina* sample 123368 was tested, the resulting DNA concentration using the PtabBM assay was 414.72 ng/µl; when the multiplex reaction was performed using the Universal Master Mix (recommended to perform the PtabBM single reaction), the DNA concentration was reduced to 272.07 ng/µl; and, when the multiplex reaction was performed using the Gene Expression Master Mix (recommended to perform the multiplex reaction using both primers/probe assays), the concentration was even lower, 48.49 ng/µl. The use of a multiplex reaction, where two sets of primers and probes are used, reduced the amount of master mix that can be utilized by the PtabBM assay itself, and the DNA concentration reported by the assay was reduced as well. When the multiplex reactions were used to diagnose *P. tabacina,* the assay was not affected significantly; but when quantification assays were performed, it is recommended to use each set of primers/probe in separate reactions.

Optimization of the PtabBM TaqMan assay. Positive reactions were detected sooner at the higher concentration of primer and probe (900 nM primers and 250 nM probe; Fig. 2A) and the slope was higher than when the lower concentrations of primer and probe were tested. The Ct value was 16.17 for the 900 nM primers and 250 nM probe, and a Ct value of 20.3 for the lowest concentration (125 nM primers and 31.2 nM probe) was obtained.

Sensitivity of the PtabBM TaqMan assay. The detection limit for *P. tabacina* was improved by the use of the two higher concentrations of primers and probe. Concentration values of 900 nM primers and 250 nM probe were useful for detection of DNA concentrations between 10 ng (Ct value of 16.17) and 10 fg (Ct value

of 37.92) (Fig. 2B). The lower concentrations of 450 nM primers and 125 nM probe were useful for detection of DNA concentrations between 10 ng (Ct value of 17.32) and 100 fg (Ct value of 36.2) of DNA (Fig. 2B). Concentrations of 450 nM primers and 125 nM probe were chosen to perform most of the subsequent assays because the quality, detection, and reliability of the reactions were optimum and the cost of the detection assay was reduced by diluting reagents. The higher concentrations of primer and probe detected lower concentrations of pathogen DNA (10 ng to 10 fg) than the lower concentrations of primer and probe (10 ng to 100 fg).

Highly reproducible Ct values with very small standard deviations were observed at a range of *P. tabacina* concentrations, and a linear response from 10 ng to 10 fg of DNA (Ct values between 19.0 and 37.0), with a detection limit of 1 fg and a late Ct value of 39 (Fig. 3A), were found. The regression equation for the DNA standard curve (Fig. 3B) was $y = -2.91 \log(x) + 22.50$, with an R^2 value of 0.99 and an efficiency value of 120%. Each 10-fold difference in initial DNA amounts was represented by approximately three cycle differences in Ct value.

Specificity of the PtabBM TaqMan assay. The detection of *P. tabacina* was highly specific using the PtabBM TaqMan primers Ptab2F and Ptab2R and the PtabBM probe. When the assay was tested against DNA from 12 other tobacco pathogens and 5 other Oomycetes, no cross-amplification by the PtabBM TaqMan assay was observed (Fig. 4). No false-positive or false-negative results were observed in any of the repetitions of the assay.

Only *P. tabacina* DNA showed a Ct value in the optimal range and the other species were not detected before 35 cycles. Isolates of *P. tabacina* had Ct values ranging from 15.0 to 35.0, representing DNA concentrations of 1,805 ng/µl to 7.53×10^{-6} ng/µl. Controls with no template were negative.

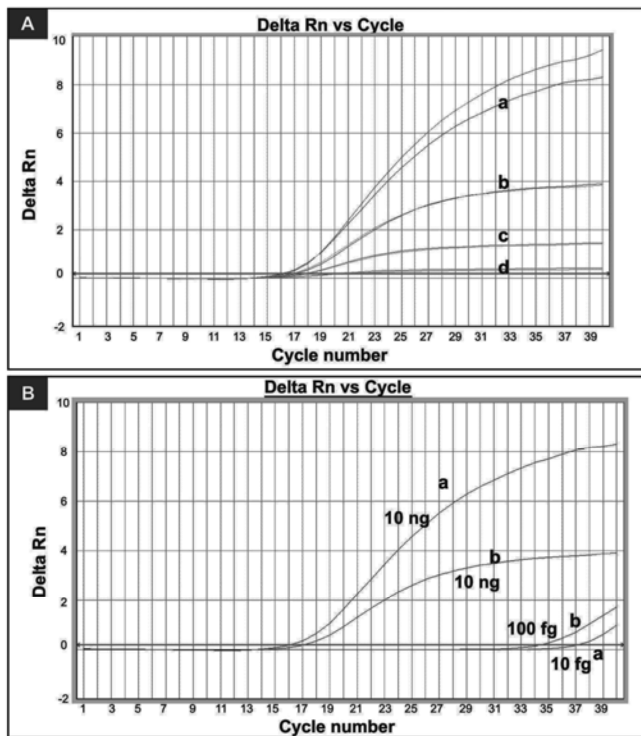

Fig. 2. Plots of the sensitivity of the primers and probe for detection of *Peronospora tabacina*. **A,** Concentrations of a, 900 nM primers and 250nM probe; b, 450 nM primers and 125 nM probe; c, 225 nM primers and 62.5 nM probe; and d, 112 nM primers and 31.2 nM probe. Cycle threshold (Ct) values are shown for duplicated samples of *P. tabacina* and were similar for all of the concentrations used. The plateau of the curve for fluorescence was affected by the different concentrations. **B,** DNA detection limit at the two highest concentrations of primer and probe is shown for a, 900 nM primers and 250 nM probe and b, 450 nM primers and 125 nM probe.

Detection of *P. tabacina* in infected field samples and artificially inoculated leaves, seedlings, and roots. Pathogen DNA from both sporangiospore solutions and infected leaves were detected with the CTAB-extracted DNA by the PtabBM TaqMan assay (Fig. 5). The real-time PCR assay worked equally well with fresh or dried leaf lesions. Sporangiospores washed from infected leaves were also detected at latter Ct values with the assay. A simple NaOH/Tris extraction method used in a previous study (40,41) was also tested and worked well with the real-time assay for detection of *P. tabacina* in infected leaves (*data not shown*; 40).

The assay was also positive for detection of *P. tabacina* extracted from DNA from field samples, and DNA concentrations from 5×10^{-5} ng/mg to 2,760 ng/mg of tissue were found. In plants with early-stage infection with chlorotic symptoms only, DNA concentrations of 4.6×10^{-6} ng/mg to 1.42×10^{-3} ng/mg of tissue were observed whereas, for late-stage lesions with the presence of sporulation, chlorosis, and necrosis, DNA concentrations of 3.8×10^{-5} ng/mg to 5.67 ng/mg of tissue were observed (Table 3). Tests on leaves with dried lesions that were stored for long periods (4 years or less) were also positive and DNA concentrations of 5.5×10^{-5} ng/mg to 68.0 ng/mg were observed.

Plants inoculated with *P. tabacina* to mimic the systemic infection that occurs naturally in tobacco transplants were also positive. Ct values between 15.3 and 16.8 and DNA concentrations of 1,863 to 2,260 ng/µl were found in older leaves and stems (1 cm adjacent to the inoculation point; Table 3). Detection of the pathogen in the newer leaves of the systemically infected tobacco plants was negative.

Roots taken 1 month after inoculation from plants with aerial symptoms of blue mold were also positive in 10 of 10 samples; Ct

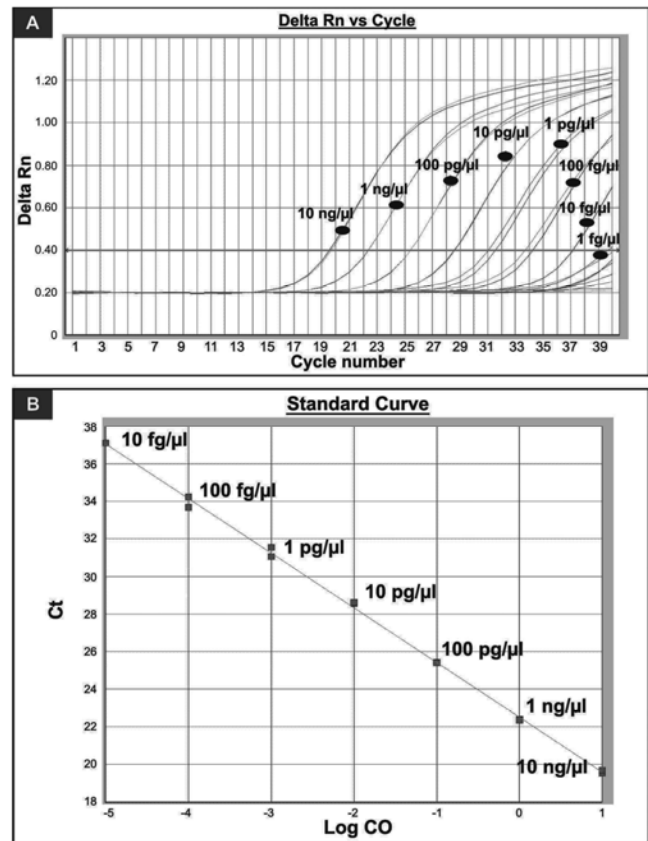

Fig. 3. Sensitivity of the PtabBM real-time assay shown in a 10-fold dilution series. **A,** Plot of ΔRn versus cycle number. The highest DNA concentration detected was at 10 ng/µl and a cycle threshold (Ct) of 19.4 and the lowest DNA concentration detected was at 1 fg/µl and of Ct = 39.8 using a concentration of 900 nM primers and 250 nM probe. **B,** Plot of Ct value versus log of concentration rate of *Peronospora tabacina* DNA. The standard curve had a slope = –2.91, intercept = 22.50, and R^2 = 0.99.

values ranged from 18.25 and 25.57 and DNA concentrations ranged from 12.60 to 155 ng/µl. Highest DNA concentrations were found in fresh lesions from field samples and systemically infected seedlings and roots.

PtabBM TaqMan assay to quantify host resistance. Two resistant burley cultivars, NC2000 and NC2002, exhibited small necrotic flecks in response to *P. tabacina* 6 days after inoculation with the pathogen. Lower DNA concentrations and less colonization by the pathogen occurred at this initial stage in NC2000 an NC2002. Ten days after inoculation, the pathogen was sporulating on both cultivars. The resistant Chemical Mutant had the lowest DNA concentration of *P. tabacina* and there was no sporulation on the discs or necrotic lesions observed. In contrast, the susceptible KY14 had the highest concentrations of DNA of the pathogen, and sporulation occurred 8 days after inoculation.

Colonization by *P. tabacina* of Chemical Mutant and KY14 was analyzed over time. Final concentrations of DNA in leaf discs were significantly different between the cultivars ($P < 0.0001$; Fig. 6). Susceptible KY14 had higher pathogen DNA concentrations after 4 days, and the pathogen increased exponentially up to 10 days after inoculation. The medium-resistant Chemical Mutant had lower pathogen DNA concentrations, possibly due to the hypersensitive response and limited colonization. At 10 days postinoculation, there were no visible symptoms of the disease in Chemical Mutant but the DNA from the pathogen was detected in lesions (Fig. 6).

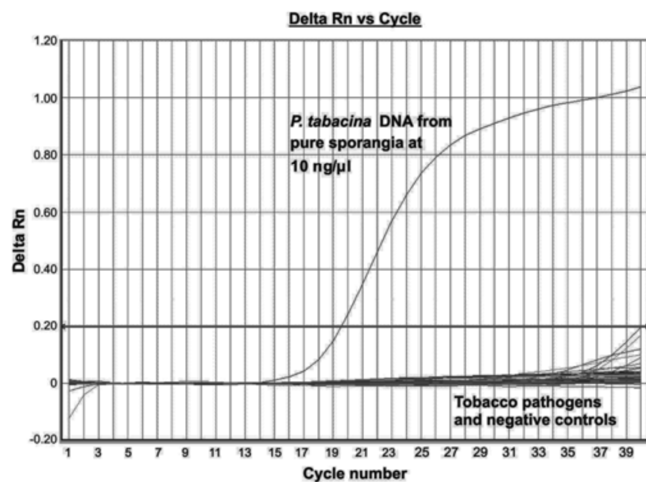

Fig. 4. Plot of the ΔRn versus cycle number of the PtabBM real-time polymerase chain reaction assay for detection of DNA from *Peronospora tabacina* sporangiospores (10 ng/µl) and DNA from 12 other tobacco pathogens, including four *Pythium* spp., two *Phytophthora* spp., one *Alternaria* sp., one *Cercospora* sp., one *Thielaviopsis* sp., one *Rhizoctonia* sp., one *Sclerotium* sp., and one *Sclerotinia* sp., and DNA from five other Oomycete spp., including *Pseudoperonospora humuli*, *Hyaloperonospora parasitica*, *Pseudoperonospora cubensis*, *Phytophthora infestans*, and *P. ramorum* tobacco pathogens and negative controls.

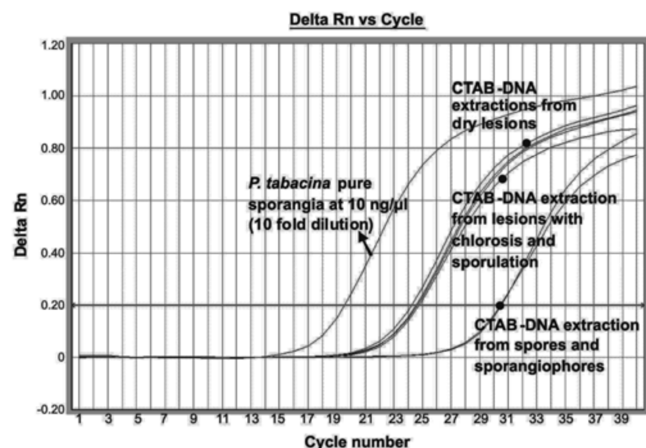

Fig. 5. Plot of ΔRn versus cycle number. Detection of *Peronospora tabacina* by real-time PtabBM polymerase chain reaction from a standard sporangiospore suspension (10 ng/µl) or fresh tobacco leaf lesions with sporulation, dried leaf lesions, and sporangiospores washed from fresh lesions. Each sample is represented by two lines (duplicates).

Discussion

P. tabacina is difficult to identify in systemic, asymptotic infections. We developed a real-time TaqMan-based method for the detection and quantification of *P. tabacina* in symptomatic and asymptomatic tissue. The PtabBM TaqMan assay is based on a multiplex PCR reaction with two universal primers, two primers for the pathogen, and two probes to monitor the identification, quantification, and quality of the DNA extracted from infected tissue.

The detection of *P. tabacina* using the quantitative PtabBM TaqMan assay was more sensitive than use of Ptab and ITS4 primers alone and conventional PCR (29). The TaqMan method was improved by the use of primers and probe generated inside a conserved region of the 5.8S rDNA. Primers Ptab2F and Ptab2R should not be used alone in conventional PCR because they share sequence homology with a number of other oomycete pathogens and are not specific in conventional PCR. The Ptab and ITS4 primers can be used for conventional PCR and are specific for *P. tabacina*. Nuclear encoded rDNA provides an attractive target because this region of DNA is highly stable, possesses conserved as well as

Table 3. Cycle threshold values (Ct) and lowest and highest concentrations of DNA detected for field samples and different tissue samples inoculated with *Peronospora tabacina*

	Ct value range[a]		DNA concentration (ng/µl) per 10 mg of tissue	
Sample[b]	Lower	Higher	Minimum	Maximum
Field leaf sample	15.2	35.4	5×10^{-5}	2,760
Chlorotic leaf	30	33	4.6×10^{-6}	1.42×10^{-3}
Sporulating leaf	24	32	3.8×10^{-5}	5.67
Dry leaf (2 mg)	18	36.4	5.5×10^{-5}	68
Systemic seedlings	15.3	16.8	1,863	2,260
Roots (15 mg)	18.25	25.57	12.6	155

[a] Ct value is the polymerase chain reaction (PCR) cycle number at which a statistically significant increase in fluorescence generated can be detected or a fluorescent signal significantly above the background fluorescence. At the Ct, a detectable amount of amplicon product has been generated during the early exponential phase of the PCR reaction.

[b] Number of samples used = 10 in all assays except sporulating lesions, where 25 samples were used.

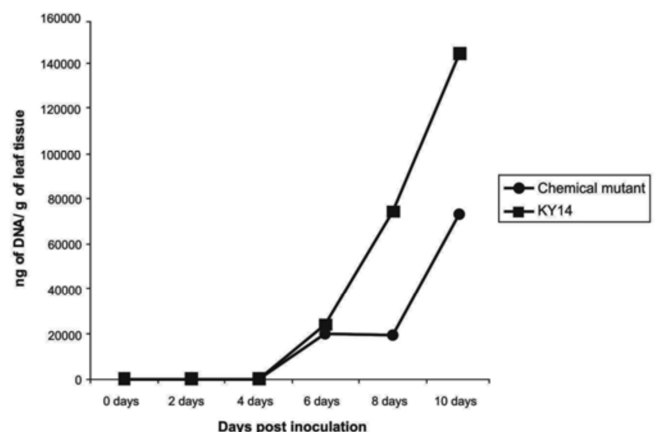

Fig. 6. Detection of DNA by real-time polymerase chain reaction from *Peronospora tabacina* in 'KY14' (susceptible burley cultivar) and 'Chemical mutant' (medium resistant flue-cured cultivar) 2, 4, 6, 8, and 10 days after inoculation.

variable sequences, and can be amplified and sequenced with universal primers. The rDNA is a multiple-copy region of genomic DNA. Multinuclear sporangiospores of *P. tabacina* have from 4 to 35 nuclei per sporangium and this increases the amount of available DNA that can be detected. (36,37).

It was possible to detect *P. tabacina* DNA at concentrations as low as 10 fg. Higher detection limits have been reported for a range of other plant pathogens, with primers developed on multi-copy genes such as the ITS region of the rDNA and conventional PCR (1,11,12,31,32). The low detection limit demonstrated the higher sensitivity of the TaqMan assay as a diagnostic tool for *P. tabacina* compared with conventional PCR (29).

We optimized the PCR reactions using serial dilutions of target DNA. Linear responses of the amount of pathogen DNA and Cts for the target pathogen were achieved. Detection limits of the assay and correlation coefficients were not affected by the presence of tobacco and coextracted plant DNA extracts. These results indicate that the method developed is appropriate for both qualitative and quantitative analyses of pathogen biomass in infected tissue.

The PtabBM TaqMan assay was specific for *P. tabacina* and did not detect many other different foliar and soilborne tobacco pathogens. The PtabBM TaqMan primers and probe were species specific and the assay showed an increase in fluorescence exclusively for isolates of the target pathogen and did not cross-react with *Pseudoperonospora cubensis*, *P. humuli*, or any other tobacco pathogens tested. All the *Peronospora tabacina* samples tested showed Ct values between 15.0 and 35.0, which is considered optimal for pathogen diagnostic assays.

The PtabBM TaqMan method was capable of detection of the presence of *P. tabacina* in field samples and artificially inoculated leaves, seedlings, and roots. The detection limits were highest for leaves with sporulation, because the internal mycelia in colonized tissue as well as aerial sporangiophores and spores are detected. DNA detection and quantification during the development of early symptoms (chlorosis or necrosis of leaves) and from DNA extracted from stored dried leaf samples was possible. Because the assay worked well in both fresh and dried tobacco tissue, it may be a useful tool for detection of the pathogen in exported tobacco leaves.

Detection of the pathogen in systemically infected tobacco plants could be a very useful tool for producers, industry, and extension agents. The detection assay allows the detection of the pathogen even before the occurrence of field symptoms, which will enable control strategies to be deployed more effectively.

The detection of *P. tabacina* in the root system a month postinoculation indicates that the pathogen is able to survive in root material for long periods of time. There are two possible explanations for this result: the presence of vegetative hyphae inside the root tissue (14) or the presence of oospores or other kinds of survival structures in the roots. It is possible that mycelia colonize the roots and remain viable once the plant dies. However, further experiments will be necessary to determine the role of root inoculum in the disease cycle.

The real-time assay might be useful to screen for the presence of oospores in tobacco roots and stems. Oospores of *P. tabacina* have been reported on in vitro plants with sporulation induced under laboratory conditions (14). In tobacco fields, the presence of oospores has not been reported since 1997 in the United States and only at very low levels of (20,33). The role of oospores in the survival of *P. tabacina* is unclear. Because oospores are difficult to germinate, screening root tissue by PCR may be useful to look for pathogen survival structures in roots. Further work is necessary to learn more about how the pathogen survives in root systems, including microscopic examination of roots for survival structures, storage of roots over longer periods of time, and other stressful conditions, including variable temperature and humidity.

Quantitative differences in colonization of tobacco tissue of moderately resistant and susceptible cultivars was documented. The TaqMan assay may be useful to tobacco breeders who are interested in developing quantitative resistance to the pathogen (25,30). The PtabBM TaqMan assay was useful for detection of *P. tabacina* 4 to 5 days postinoculation, before symptoms occurred. Colonization of the pathogen inside moderately resistant cultivars was not completely suppressed, even though there was no sporulation externally on plants. These results indicate that, if moderately resistant tobacco cultivars are planted in the field, survival of the pathogen is still possible in infected asymptomatic tissue.

The real-time method described here gives an accurate, reliable, and highly efficient procedure for quantification of the target pathogen DNA in infected tissue. The ability to quantify the amount of DNA present in a symptomatic or nonsymptomatic lesion using the PtabBM TaqMan assay will contribute to the improved diagnostics of this important plant pathogen. In many years, the pathogen has spread to noninfested areas of the United States in infected tobacco transplants. Screening tobacco transplants for the presence of the pathogen before shipment might be useful to reduce the likelihood of pathogen spread. Such screening could be done by plant diagnostic clinics. The need for such a test has been increased over the last few years with the spread of the pathogen from one tobacco-producing area in the United States to another via shipments of infected transplants or the spread of the pathogen on wind currents in the American and European continents.

Potential disadvantages of the method include cost and the need for well-equipped laboratories that have real-time PCR machines. Also, the inability to determine whether the target organism is viable could be an issue because the assay detects DNA and not viable propagules of the pathogen. The risk of obtaining false positives and negatives can never be fully discounted due to the inability to fully assess the biodiversity of other species of *Peronospora* (4).

The assay has great potential as a tool for identification and detection in a wide range of applications from pathogen surveys to statutory testing. This method offers advantages over conventional PCR procedures and will provide a useful and rapid tool in nationwide efforts to detect *P. tabacina*. There is also a great interest in world markets for tobacco exports of leaves, transplants, and seed. There is concern about the potential movement of *P. tabacina* with these materials to some countries where the pathogen may not occur. The sensitivity, accuracy, and rapidity of this PCR-based technique could be valuable to national regulatory agencies attempting to help prevent the spread of *P. tabacina* and subsequent disease.

Acknowledgments

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