Plant Pathology Journal

ISSN 1812-5387

science alert

ANSInet
an open access publisher
http://ansinet.com

Plant Pathology Journal 5 (3): 378-382, 2006
ISSN 1812-5387

Specific Detection of *Peronospora tabacina* by PCR-amplified rDNA Sequences

[1]Jwu-Guh Tsay, [1]Chishih Chu, [2]Ya-Han Chuang and [2]Ruey-Shyang Chen
[1]Department of Applied Microbiology,
[2]Graduate Institute of Biotechnology, National Chiayi University, Chiayi 600, Taiwan

Abstract: *Peronospora tabacina* Adam, a downy mildew fungus, is a devastating disease of tobacco and a pathogen of import prohibition in Taiwan. For quarantine purpose, we developed a nested PCR method to detect this pathogen. With universal primer pair ITS1/ITS4, internal transcribed spacer (ITS) region of pathogen's rDNA was amplified. Specific PCR primers (PT1/PT2) were designed based on ITS sequence and used to amplify a 493-bp rDNA fragments from *P. tabacina*. In order to increase the sensitivity, another primer pair (PT3/PT4) was used in a one-tube nested PCR. The expected 243-bp fragment was amplified from *P. tabacina*, but not from any other tested fungi or commercial tobacco leaves. The detection limit of this nested PCR could be low as 1 pg of template DNA. The present nested PCR method showed to be rapid, simple and available as a tool to screen *P. tabacina* infection for quarantine purpose.

Key words: Tobacco, downy mildew, nested PCR, internal transcribed spacer

INTRODUCTION

Downy mildews are primarily foliage blights that attack young, tender leaves, twigs and fruits. It spreads rapidly through fields and is dependent on a wet, humid environment with cool or warm, but not hot, temperatures to grow and infect. Several different fungi cause downy mildew disease including *Peronospora* (mildew on spinach, soybeans, onion, many ornamentals), *Plasmopara* (on grape), *Bremia* (on lettuce), *Psuedoperonospora* (on curcurbits), *Peronosclerospora* (on corn) and *Sclerospora* (on grasses) (Agrios, 1997). Downy mildew of grape, cucurbit and crucifer vegetables cause serious economic losses in Taiwan (Tsai, 1999). Recently, downy mildew was found not only in leaves but also in pods and seeds of vegetable soybeans in spring season and severely reduced the quantity and quality of vegetable soybeans (Lai *et al*., 2004).

Blue mold of tobacco (also known as 'mildiou du tabac' in Europe), caused by Peronospora tabacina Adam, is a classical compound-interest plant disease that occurs locally as well as macroscale epidemically (McKeen, 1989), but this downy mildew fungus of tobacco was a pathogen of import prohibition in Taiwan. Since *P. tabacina* was an obligately parasitic oomycete, a number of molecular markers have been applied to investigate genetic diversity in *P. tabacina*, including isoenzymes (Edreva *et al*., 1998), internal transcribed spacer sequences (Wiglesworth *et al*., 1991) and random amplified polymorphic DNA (RAPD) markers (Wiglesworth *et al*., 1994). Especially, ribosomal internal transcribed spacer (ITS) regions were found to accommodate more interspecific variations and provide attractive targets for phylogeny studies as well as molecular detection of fungi (Lee and Taylor, 1992; Matsumoto *et al*., 1997). The universal ITS primers designed by White *et al*., (1990) have been used extensively in detection of various fungal pathogens in Taiwan, such as *Pythium* sp. (Wang and while, 1997; Wang and chang, 2003; Wang *et al*., 2003), *Peronospora manshurica* (Lie *et al*., 2004) and *Ustilago esculenta* (Chen and Tzeng, 1990). To use the advantages of nucleotide variations in ITS region, we developed specific primers to detect the pathogen *P. tabacina* by nested PCR. This PCR procedure is expected to be adapted for quarantine purpose.

MATERIALS AND METHODS

Fungal isolation and identification: Leaves of downy mildews-infecting vegetable soybean (*Glycine max* Merrill), cucumber (*Cucumis sativus* L.) and rape (*Brassica napus* L.) were collected in 2003. The pathogens causing such infection were examined and identified according to their leaf symptoms, sporangiophores and sporangia (Agrios, 1997; Lai *et al*., 2004). Most of these pathogens were fungi including *Peronospora manshurica* (Naoum.) Syd. ex Gaum., *P. parasitica* (Pers.: Fr.) Fr., *Plasmopara viticola*

Corresponding Author: Ruey-Shyang Chen , Graduate Institute of Biotechnology, National Chiayi University, Chiayi 600, Taiwan
Tel: 886-5-2717897 Fax: 886-5-2750396

1	AGGTACATTA	CCACACCTAA	AAACATTCCA	CGTGAACCGT	ATCAACCCCA
	18S rDNA	ITS1			
51	AGTTGGGGGT	TTCATTGGCG	GCGGCTGCTG	GCATCTTTTT	GCTGGCTGGC
101	TACTGCTGAG	AGAACCCTAT	CGTGAGCGTT	CTGACCTTGG	TTGGAGCTAG
			Primer PT1		
151	TAACTTATTA	TAAACCCATT	CCTAATACTG	AATATACTGT	GGGGACGAAA
				Primer PT3	
201	GTCTCTGCTT	TTAACTAGAT	AGCAACTTTC	AGCAGTGGAT	GTCTAGGCTC
			5.8S rDNA		
251	GCACATCGAT	GAAGAACGCT	GCGAACTGCG	ATACGTAATA	CGAATTGCGG
301	AATTCAGTGA	GTCATCGAAA	TTTTGAACGC	ATATTGCACT	TTCGGGTTAT
351	CCCTGGAAGT	ATGCCTGTAT	CAGTGTCCAT	ACATCAAACT	TGGTTTTCTT
				ITS2	
401	CTTTCCGTGT	AGTCGGTGGA	GGATATGCCA	GATGTGAAGT	GTCTTGCGGC
		Primer PT4			
451	TGGTTTTTTT	GAATCGGCTG	CGAGTCCTTT	GAAATGTATG	GAACTGACTC
501	TCTTTTGTTC	GAAAAGCGTG	GCGTTGCTGG	TTGTGAAGGC	TGCTAGTATG
551	ACTAGTCGGC	GACCGGTTTG	TCTGCTATGG	CATGAATGGA	AGAGTGTTCG
					Primer PT2
601	ATTCGCGGTA	TGGTTGGCTT	CGGCTGAACA	GGCGCGTATT	GGACGTTCAT
651	CCCGCTGTGG	CGTATAACTG	GTGAACCGTA	GTTCATGCAT	GGCTTGGCGT
701	TTGAATCAGC	TTTGCTGCTG	CGAAGTAGAG	TGACAGTTTT	GGCTGTCGAG
751	GGTCGACCTA	TTTGAGAAAT	TGTGCTGTGC	GACTTCGGTC	GCGTGGCATC
801	TCAATTGGAC	CTGATAACAG	GCAAGAT		
		28S rDNA			

Fig. 1: Nucleotide sequence and primer location of ITS region amplified by primers ITS1/ITS4 from *Peronospora tabacina* KY-Bo-99

(Berk and M.A. Curtis) Berl and De Toni in Sacc., *Pseudoperonospora cubensis* (Berk. and M.A. Curtis) Rostovzev, two anthracnose fungi of vegetable soybean: *Glomerella cingulata* (Stoneman) Spauld. and H. Schrenk and *Colletotrichum truncatum* (Schwein.) Andrus and W.D. Moore, *Fusarium solani* (Mar.) Sacc. f.sp. *cucurbitae* W.C. Snyder and H.N. Hans. a pathogen of crown and foot rot of melon and *Botrytis elliptica* (Berk.) Cooke - a pathogen of gray mold of lily.

DNA preparation: DNA isolation was carried out using a kit protocol provided by DNeasy Plant Mini Handbook (Qiagen Inc., Valencia, CA). DNA sample of *Peronospora tabacina* PA 98 and KY-Bo-99 isolates by courtesy of Dr. Mark L. Farman (Sukno *et al*., 2002a, b), Department of Plant Pathology, University of Kentucky, USA. Six brands of commercial tobacco, Long Life Mild, New Paradise Mild and Prosperity Island Light (Taiwan Tobacco and Liquor Co.), Marlboro, Mild Seven and 555, were collected from a supermarket. One hundred mg of dry tobacco leaves was used for DNA extraction by using the Qiagen DNeasy Plant Mini kit (Qiagen Inc., Valencia, CA). The final DNA was dissolved in 50 μL of TE buffer and 5 μL aliquots of 1:10 DNA dilutions were used for the nested PCR amplifications.

PCR condition: PCR reaction mix contained 0.1 mM dNTPs, 0.25 μM primers, 1 U Bio-Thermal DNA Polymerase, 1 X PCR buffer with 1.5 mM $MgCl_2$ and 10 ng of template DNA. Sterile distilled water was added to reach the final volume of 25 μL. PCR amplification was carried out with a thermal cycler (GeneAmp PCR System 2400, Perkin-Elmer, Applied Biosystem Division, Norwalk, CT). The running condition for primer set (ITS1/ITS4) were 1 min at 94°C for initial denaturation followed by 30 cycles consisting of 1 min of denaturation at 94°C, 1 min of annealing at 48°C and 1 min of DNA synthesis at 72°C (Chen and Tzeng, 1999). An extension time of 10 min at 72 C was added at the end of the last cycle. For the nested PCR primer sets, the conditions changed from previously PCR running condition were 45 sec of annealing at 55°C and 30 sec of extension for primer set (PT1/PT2) and 30 sec of annealing at 67 C for primer set (PT3/PT4). Amplified product of DNA was separated in 0.5% TBE agarose gel and stained with ethidium bromide. Sequence of the amplified product was analyzed using an automated sequencer at the Minsheng Biotechnology Co. (Taipei, Taiwan).

RESULTS AND DISCUSSION

To ensure the specific detection of *P. tabacina*, not related species or downy mildew pathogens, ITS region containing part of 18S rRNA, ITS1, 5.8S rRNA, ITS2 and part of 28S rRNA was selected as detection target. The 827-bp DNA fragment amplified from *P. tabacina* was sequenced (Fig. 1) and aligned with other published ITS sequences. Based on the sequence

Table 1: Primer sequences used to amplify the ITS region of the fungal species

Primer	Sequence	Annealing temperature(°C)	PCR product (bp)	Reference
ITS-1	5'-TCCGTAGGTGAACCTGCGG-3'	48	827	White *et al.* (1990)
ITS-4	5'-TCCTCCGCTTATTGATATGC-3'			
PT1	5'- ATCGTGAGCGTTCTGAC CTT -3'	55	493	This study
PT2	5'- ATACCGCGAATCGAACA CTC -3			
PT3	5'-CTGTGGGGACGAAAGTCTCT-3'	67	243	This study
PT4	5'-GGCATATCCTCCACCGACTA-3			

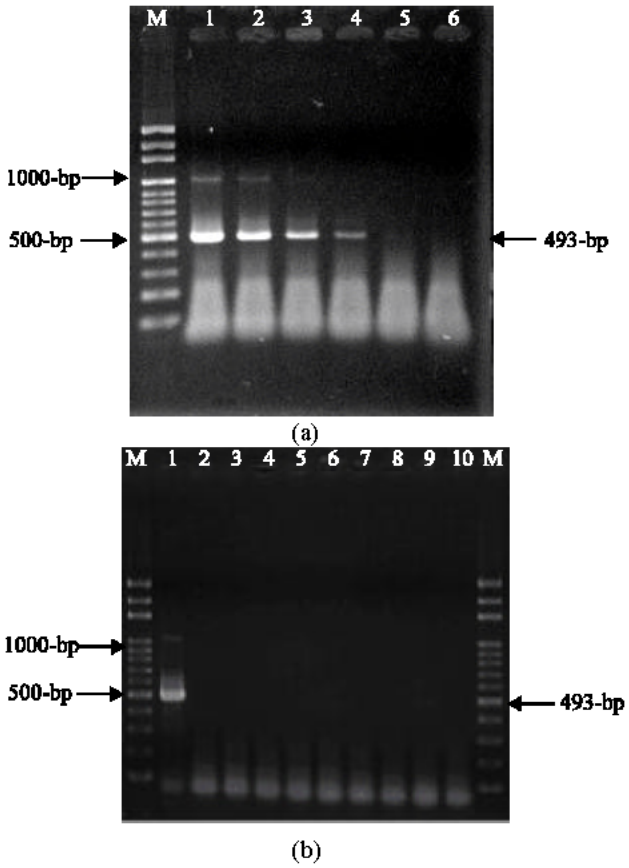

Fig. 2: Primers PT1/PT2-amplified species-specific PCR product (493-bp) for *P. tabacina*. 2a. Sensitivity test of primers PT1/PT2 with purified DNA of *P. tabacina*. Lane M, DNA marker (Bio 100 DNA ladder); lane 1 to 5: DNA concentration of *P. tabacina* at 10 ng μL^{-1}, 1 ng μL^{-1}, 100 pg μL^{-1}, 10 pg μL^{-1}, 1 pg μL^{-1} of DNA; lane 6: negative control. 2b. Specificity test of primers PT1/PT2 from DNA of six commercial tobacco brands. Lane M, DNA marker (marker sizes are indicated in kb); lane 1, *Peronospora tabacina* 10 ng μL^{-1}; lane 2, healthy tobacco leaves; lane 3, Long Life Mild; lane 4, New Paradise Mild; lane 5, Prosperity Island Light; lane 6, Mild Seven; lane 7, 555; lane 8, Marlboro; lane 9, New Paradise Mild + *P. tabacina* 1 pg μL^{-1}; lane 10, negative control

differences, primer pair PT1/PT2 which was specific to *P. tabacina* were designed (Table 1). A product of 493-bp was amplified with the primer pair PT1/PT2 with a minimal DNA concentration of 10 Pg μL^{-1}, only from *P. tabacina* (Fig. 2a), but not from related species *P. manshurica and P. parasitica* and other downy mildew fungi *Pseudoperonospora cubensis*, *Plasmopara viticola* and *Glomerella glycines*, *Colletotrichum truncatum*, *Fusarium solani* f. sp. *cucurbitae* and *Botrytis elliptica* (data not shown).

This primer set PT1/PT2 showed high specificity and high sensitivity to detect *P. tabacina*. However, whether this primer set could cause false positive result, a mixture of DNA samples of *P. tabacina* with tobacco leaves and six commercial brands of tobacco were tested. No PCR product was found from these tobacco leaves, except the sample mixed with *P. tabacina* DNA (Fig. 2b). Certain DNA preparations with DNA contamination from a source other than *P. tabacina* were detected in the spore suspensions (Sukno *et al.*, 2002b). To ensure the specific detection of *P. tabacina*, the internal nested PCR primer set (PT3/PT4) was tested (Table 1). The sensitivity of *P. tabacina* to the one-tube nested PCR was 1 pg (~2 copies) of DNA without false positive result (Fig. 3).

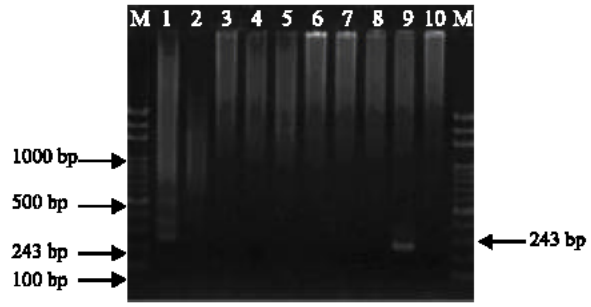

Fig. 3: 243-bp amplified of DNA fragments from *P. tabacina* using one-tube nested PCR with primer sets PT1/PT2 and PT3/PT4. Lane M, DNA marker (marker sizes are indicated in kilobases); lane 1, *P. tabacina* 10 ng μL^{-1}; lane 2, healthy tobacco leaves; lane 3, Long Life Mild; lane 4, New Paradise Mild; lane 5, Prosperity Island Light; lane 6, Marlboro; lane 7, Mild Seven; lane 8, 555; lane 9, New Paradise Mild + *P. tabacina* 1 pg μL^{-1}; lane 10, negative control

Although genetic variability may be lacking in *P. tabacina* populations, many physiologic races of *P. tabacina* have been identified in the United States on the basis of disease reactions of differential cultivars (Sukno *et al*., 2002a, 2002b; Lucas, 1980). Recently, molecular methods or a number of molecular markers, including isoenzymes (Edreva *et al*., 1998), internal transcribed spacer sequences (Wiglesworth *et al*., 1991) and random amplified polymorphic DNA (RAPD) markers (Wiglesworth, 1994), have been applied to investigate genetic diversity in *P. tabacina*. After random amplified polymorphic DNA (RAPD) analysis, species-specific DNA fragment may be selected from the amplification products. Primers are then designed from the nucleotide sequences and used to detect directly specific species with higher specificity and sensitivity. This approach has been used to develop PCR methods for detection of *Peronospora tabacina* on tobacco (Wiglesworth, 1994).

Applications of the molecular technologies has greatly improved our ability to detect and monitor plant fungal pathogens, including increased sensitivity, reduction of time required for testing and better chance to differentiate complex infection as well as fungal strains. *Peronospora tabacina* Adam, a downy mildew fungus, is a devastating disease of tobacco and has currently not been found in Taiwan. For quarantine purpose to prevent the invasion of this pathogen through imported vegetables, the present PCR-based detection method was developed for identification of *P. tabanina* directly from total DNA extracts of infected plants. These results suggested that this nested PCR method is rapid, simple and available as a tool to screen downy mildew, *P. tabacina,* infection for quarantine purpose.

ACKNOWLEDGMENTS

This study was supported by the grant No. 91AS-7.2.2-BQ-B15 from Bureau of Animal and Plant Health Inspection and Quarantine, the Council of Agriculture, Taiwan, R.O.C. Dr. Mark L. Farman, Department of Plant Pathology, University of Kentucky, USA helping to provide DNA samples of *Peronospora tabacina* for conducting this research, is greatly appreciated.

REFERENCES

Agrios, G.N., 1997. Plant Pathology. 4th Edn., Academic Press, New York, pp: 635.

Chen, R.S. and D.D. Tzeng, 1999. PCR-mediated detection of *Ustilago esculenta* in wateroat (*Zizania latifolia*) by ribosomal internal transcribed spacer sequences. Plant Pathol. Bull., 8: 149-151.

Edreva, A., R. Delon and J. Coussirat, 1998. Variability of *P. tabacina* A. An isoenzyme study. Beitr. Tabakforsch. Intl., 18: 3-13.

Lai, P.S., T.J. Lien, R.S. Chen and J.G. Tsay, 2004. The occurrence of downy mildew of vegetable soybean and its detection by PCR. Plant Prot. Bull., 46: 155-162.

Lee, S.B. and J.W. Taylor, 1992. Phylogeny of five fungus-like protoctistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. Mol. Biol. Evol., 9: 636-653.

Lucas, G.B., 1980. The war against blue mold. Science, 210: v147-153.

Matsumoto, C., K. Kageyama, H. Suga and M. Hyakumachi, 1999. Phylogenetic relationships of *Pythium* species based on ITS and 5.8S sequences of the ribosomal DNA. Mycoscience, 40: 321-331.

McKeen, W.E., 1989. Blue Mold of Tobacco. APS press. St. Paul, Minn, USA., pp: 288.

Tsai, Y.P., 1991. List of Plant Diseases in Taiwan 3rd Edn. The Plant Protection Society, Taiwan, R.O.C. pp: 604 (in Chinese)

Wang, P.H. and J.G. White, 1997. Molecular characterization of *Pythium* species based on RFLP analysis of the internal transcribed spacer region of ribosomal DNA. Physiol. Mol. Plant Pathol., 51: 129-143.

Wang, P.H. and C.W. Chang, 2003. Detection of the low-germination-rate resting oospores of *Pythium myriotylum* from soil by PCR. Lett. Applied., Microbiol., 36: 157-161.

Wang, P.H., Y.T. Wang and J.G. White, 2003. Species-specific PCR primers for *Pythium* developed from ribosomal ITS1 region. Lett. Applied Microbiol., 37: 127-132.

White, T.J., T. Bruns, S. Lee and J. Taylor, 1990. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: PCR Protocols: A Guide to Methods and Applications. Innis M.A., D.V. Gelfand, J.J. Sninsky and T.J. White (Eds.), Academic Press, New York pp: 315-322.

Wiglesworth, M.D., C.L. Schardl, W.C. Nesmith and M.R. Siegel, 1991. Differentiation of peronosporales and isolates of *Peronospora tabacina* by direct sequencing of an internal transcribed spacer (ITS2). Phytopathology, 81: 1237.

Wiglesworth, M.D., 1994. Differentiation and detection of *Peronospora tabacina* Adam. using the polymerase chain reaction. Ph.D Thesis, University of Kentucky, Lexington.

Sukno, S.A., A.M. Taylor and M.L. Farman, 2002a. Development of contamination-free restriction fragment length polymorphism probes for the obligate biotroph *Peronospora tabacina*, an oomycete causing blue mold of tobacco. Phytopathology, 92: 1227-1235.

Sukno, S.A., A.M. Taylor and M.L. Farman. 2002b. Genetic uniformity among isolates of *Peronospora tabacina*, the tobacco blue mold pathogen. Phytopathology, 92:1236-1244.