한국식물병리학회지 14(6) : 589-593(1998)
Korean J. Plant Pathol. 14(6) : 589-593(1998)

Detection of *Plasmodiophora brassicae* by Using Polymerase Chain Reaction

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PCR을 이용한 *Plasmodiophora brassicae*의 검출

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ABSTRACT: DNA amplification by polymerase chain reaction (PCR) was used to specifically detect *Plasmodiophora brassicae*, causing clubroot of crucifers. On the basis of DNA sequence informations, an oligonucleotide primer set specific for the pathogen was designed from small subunit gene (18S-like) and internal transcribed spacer (ITS) region of ribosomal DNA. Primer ITS 5/PB-C produced an amplification product of approximately 520 bp in length with DNA from *P. brassicae*. However, no amplification product was produced with DNAs from several soil-borne fungi, *Didymella bryoniae* and *Rhizopus stolonifer*. Using these primers, the clubroot pathogen was readily detected from infected roots of crucifers, but not from healthy roots. Southern hybridization analysis further confirmed that the amplification product was originated from *P. brassicae*.

***Key words*: *Plasmodiophora brassicae*, PCR, primer, southern hybridization.**

Plasmodiophora brassicae Woronin causing clubroot of crucifers is one of the most destructive soil-borne pathogens. *P. brassicae* is an obligate endoparasite of crucifers including Chinese cabbage, cabbage and radish. *P. brassicae* is classified as a member of the Plasmodiophoromycota within the Kingdom Protozoa (3) which are characterized by the production of plasmodia and resting spores in the host. When infected roots decay, resting spores are released into soil. Resting spores in soil provide the source of inoculum in natural infection and survive for a long period of time. Therefore, reliable method for detection of resting spore or plasmodium of the pathogen would be of great value in epidemiological researches and potentially for disease monitoring programs.

Takahashi and Yamaguchi (10) assessed the pathogenic activity of resting spores by fluorescence microscopy. Wakeham and White (12) developed serological method to detect resting spores of *P. brassicae*. However, detection of the pathogen by microscopic observation has a limitation and serological method is laborious and time consuming. Development of sensitive and simple technique may provide useful tool to detect this pathogen in plant tissue or soil.

Recently, PCR-based molecular markers are widely used to detect phytopathogenic organisms. Pathogen detection based on PCR has numerous advantages over conventional methods. Detection can be made from cultures or *in planta* with equal facility and very small quantities of pathogen can be detected (4). Rollo *et al.* (7) detected *Phoma tracheiphila* in infected woody tissue by PCR technique. Schesser *et al.* (9) used PCR to detect the take-all fungi in infected wheat. Chen *et al.* (2) demonstrated that PCR detection of phytoplasma is more sensitive than microscopic, serological methods. Ribosomal DNA sequence data has been widely used to design pathogen-specific PCR primers. Ribosoml DNA is considered to be suitable genomic sites for primer design because of high gene copy number and the nature of sequences containing both conserved and variable region. Nazar *et al.* (6) used ribosomal DNA specific primers to detect and differentiate isolates of *Verticillium* spp. Johanson and Jeger (5) detected *Mycosphaerella fijiensis* and *M. musicola* in banana leaf using primers synthesized from a variable

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region of ITS (internal transcribed spacer) of ribosomal DNA. Up to now, no *P. brassicae*-specific primer has been developed which would allow PCR-based diagnosis of clubroot. The objective of this study was therefore to develope a PCR-based assay for the detection and identification of *P. brassicae*. In this study, we designed a specific primer based on ribosomal DNA to detect *P. brassicae*.

MATERIALS AND METHODS

Isolates and DNA preparation. *P. brassicae* and fungi used in this study are listed in Table 1. Clubroot galls were obtained from infected Chinese cabbage, cabbage and radish. In order to harvest resting spores, clubroot galls were smashed with mortar and pestle. The samples were filtered through eight layers of cheese clothes. and the filtrate was centrifuged for 5 min at 2,500 rpm. The pellet was resuspended in 30 ml of distilled water. This centrifugation procedure was repeated twice. Final pellet was resuspended in 0.5 ml distilled water and frozen at -20°C. The samples was subsequently freeze dried for 8 hours. Freeze dried samples were ground in liquid nitrogen with mortar and pestle. The sample was resuspended in extraction buffer (25 mM Tris-HCl, 25 mM EDTA, 50 mM NaCl, 1% SDS, pH 8.0) and incubated at 55°C for 1 h. The sample was mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1), then centrifuged at 12,000 rpm for 10 min. The upper aqueous phase was recovered and the nucleic acids were precipitated by the addition of one volume of cold isopropanol. Ribonuclease was added and incubated at 4°C overnight to completely digest contaminating RNA. Fungi used in this study were identified according to morphological characters and obtained from the fungal collection of the National Institute of Agricultural Sciences and Technology at Suweon, Korea. Fungi were grown on potato dextrose broth medium at 24°C. Genomic DNA of fungi was extracted by simple and rapid method described by Cenis (1). Genomic DNA was extracted from healthy roots as well as clubroot galls of Chinese cabbage, cabbage and radish using the protocols of Rogers and Bendich (8).

Table 1. List of organisms used in this study

Organism	Source	Host
Plasmodiophora brassicae	Jinbu	Chinese cabbage
Plasmodiophora brassicae	Pyeongchang	Cabbage
Plasmodiophora brassicae	Pyeongchang	Radish
Rhizoctonia solani 950350	Jeonju	Radish
Fusarium oxysporum F6325	Buyeo	Chinese cabbage
Phytophthora capsici Pa-11	Cheongsong	Red pepper
Phytophthora cactorum Pb-9	Andong	Apple
Pythium sp.	Boryoung	Rose
Trichoderma sp. TR-014	Sangju	Soil
Penicillium sp. PE-003	Sangju	Soil
Didymella bryoniae	Yeoju	Melon
Rhizopus stolonifer	Chilgok	Red pepper
Sclerotinia sclerotiorum SS9505	Boryoung	Cucumber

PCR. Oligonucleotide primers were designed according to ribosomal DNA sequences of *P. brassicae* (13). ITS 5 primer site was selected as universal conserved sequences. In order to select primer site specific to *P. brassicae*, nucleotide sequences of the ITS region of *P. brassicae* were compared with the same region of other members of Plasmodiophoromycota including *Polymyxa, Spongospora, Ligniera* and *Olpidium* (Table 2). A 18 base region specific to *P. brassicae* with little homology to others was identified, and selected for the design of the PB-C primer. Primers were synthesized by Bioneer Inc., Seoul, Korea. Primer sequences were as follow:

ITS 5 : 5'AGAAGGAGAAGTCGTAACAAGG3'
PB-C : 5'TGGGATGCAGCGCTAGGG3'

PCR was performed using Ericomp thermocycler (Ericomp Inc. U.S.A.). Amplification reaction was carried out in 50 mM-KCl, 10 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$, containing 50 mM of each dATP, dCTP, dGTP, and dTTP, 150 nM primer, 4 unit of Taq DNA polymerase (Promega) and 50 ng of template DNA in a final volume of 40 μl. The thermocycler was programmed for one cycle of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 56°C, 2 min at 72°C, and one cycle of 5 min at 72°C. Reaction products were

Table 2. Nucleotide sequences of variable region in the ITS of ribosomal DNA

Organism	Sequences	Acession No.
Polymyxa graminis	CCATGCGAAAAACATGT	Y12824
Polymyxa betae	CCTTTGGTTAAATGTGG	Y12827
Ligniera sp.	TTCCTTTGGCTCTATGT	Y12828
Plasmodiophora brassicae	CCCTAGCGCTGCATCCCA	U18981
Spongospora subterranea	GCAGACCCCAAAA**CCA	Y12829
Olpidium brassicae	TTAACCCAAGACCTGCC	Y12830

resolved by electrophoresis on 1.5% agarose gel in 0.5 X Tris-borate-EDTA buffer and stained with ethidium bromide (10 mg/ml) for 20 min, then visualized with u. v. light.

Southern hybridization. The gel was depurinated in 0.25 M-HCl for 15 min. After rinsing twice with distilled water, the gel was denatured in 0.5 N NaOH for 30 min. The gel was placed in neutralization solution (0.5 M Tris-Cl, 1.5 M NaCl, pH 8.0). The DNA was transferred by vacuum transfer to a nitrocellulose membrane using 20×SSC. Amplified PCR fragment from PCR reaction with primer combination PB-C/ITS 5 was excised from agarose by squeezing. The fragment were then labelled by the Gene Images Random Prime Labelling Kit (Amersham). Hybridization was carried out according to the manufacturer's instruction. Signals were detected by exposing the filter to Fuji X-ray film for 3 hr.

Sensitivity of detection. In order to assess the sensitivity of PCR-detection using ITS5/PB-C primers, PCR amplification was performed with the genomic DNA of resting spore of *P. brassicae*. Serial ten-fold dilutions of the genomic DNA sample were prepared. Reactions were set up with variable quantities of genomic DNA ranging from 10 μg to 1 pg. PCR was carried out as described above. PCR products were electrophoretically separated on 1.5% agarose gel and stained with ethidium bromide (10 mg/ml) for 30 min, then visualized with u.v. light.

RESULT AND DISCUSSION

In this study, we designed specific primers to detect *P. brassicae*. Primers were designed from the region of the small subunit gene (18S-like) and internal transcribed spacer in ribosomal DNA of *P. brassicae* (Fig. 1). Ward and Adams (13) sequenced ribosomal DNA of several members of Plasmodiophoromycota including *Polymyxa, Spopngospora, Plasmodiophora, Ligniera, Olpidium*. They revealed that 480 bp insertion sequence between ITS5 and NS8 primer sites exist only in the DNA of *P. brassicae*. In order to select specific primer for *P. brassicae*, ITS regions were compared

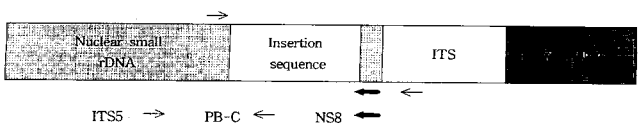

Fig. 1. Location of primers in ribosomal DNA repeat unit.

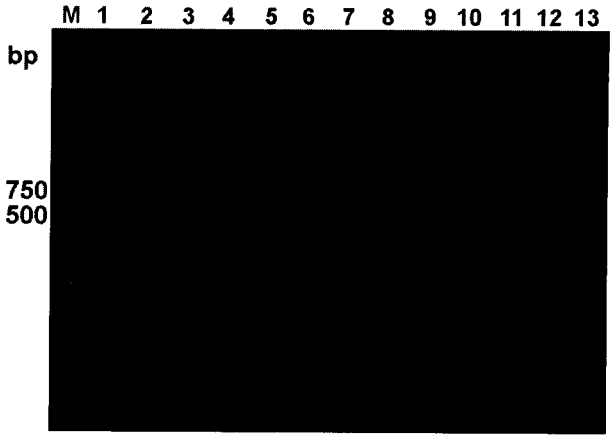

Fig. 2. Results of DNA amplification with ITS5/PB-C. M: 1 kb ladder (Promega), 1: *Plasmodiophora brassicae* from Chinese cabbage, 2: *P. brassicae* from cabbage, 3: *P. brassicae* from radish, 4: *Rhizoctonia solani*, 5: *Fusarium oxysporum*, 6: *Phytophthora capsici*, 7: *Phytophthora cactorum*, 8: *Pythium* sp., 9: *Trichoderma* sp., 10: *Penicillium* sp., 11: *Didymella bryoniae*, 12: *Rhizopus stolonifer*, 13: *Sclerotinia sclerotiorum*.

among members of Plasmodiophoromycota. PB-C primer site with little homology to the same site of other members of Plasmodiophoromycota was identified and used for PCR amplification with ITS5 primer. Designed primers successfully amplified the DNA of *P. brassicae*. PCR with primer PB-C and ITS5 amplified a product of approximately 520 bp in size (Fig. 2). The primer set amplified DNA of three isolates of *Plasmodiophora brassicae* from various hosts including Chinese cabbage, cabbage and radish. No PCR products were observed with genomic DNA from fungal species including *Fusarium, Phytophthora, Pythium, Rhizoctonia, Penicillium, Trichoderma, Sclerotinia* which are commonly found in soil (Fig. 2). No amplification products were obtained with the DNA from *Rhizopus* sp. and *Didymella* sp. These results indicate that no corresponding sites of PB-C primer exist in the genomic DNA of fungi used in this study. The PCR method was sufficiently sensitive to detect the DNA of *P. brassicae* from infected root tissue. DNA from healthy roots of radish, cabbage, Chinese cabbage was not amplified with the primers. In contrast, DNA extract from roots which showed typical symptom of clubroot was successfully amplified using the primer (Fig. 3). By southern blotting, the PCR fragment from *P. brassicae* was found to hybridize to the PCR products produced from the infected plant (Fig. 4). This result confirmed that the PCR product from the infected plant was amplified from the genomic DNA of *P. brassicae*. The

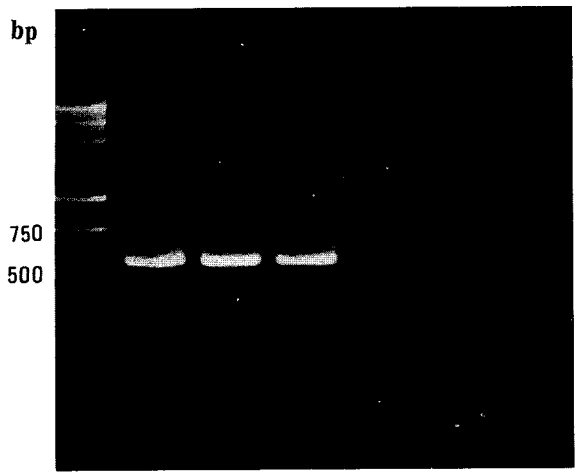

Fig. 3. Result of DNA amplification with primer ITS5/PB-C. M: 1 kb ladder (Promega) 1: clubroot of Chinese cabbage, 2: clubroot of cabbage, 3: clubroot of radish, 4: healthy root of Chinese cabbage, 5: healthy root of cabbage, 6: healthy root of radish.

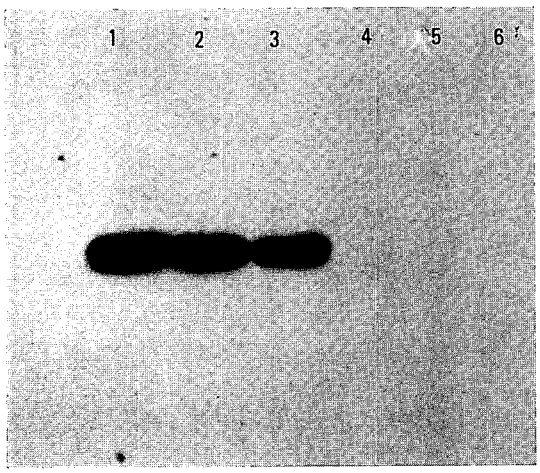

Fig. 4. Southern hybridization with the labeled PCR product. 1: clubroot of Chinese cabbage, 2: clubroot of cabbage, 3: clubroot of radish, 4: healthy root of Chinese cabbage, 5: healthy root of cabbage, 6: healthy root of radish.

sensitivity of detection of genomic DNA of *P. brassicae* by this method was high. PCR amplification with specific primers derived from ribosomal DNA regions may have an advantage of high sensitivity because of high copy number of ribosomal gene. When a serial dilution of *P. brassicae* genomic DNA as template DNA was made for the PCR amplification, successful amplification was achieved with upto 10 p.g. of DNA (Fig. 5). The intensity of bands was in direct proportion to the amount of template DNA used in the PCR. In order to extract DNA from resting spores, it is very important to select the effective method of disrupting

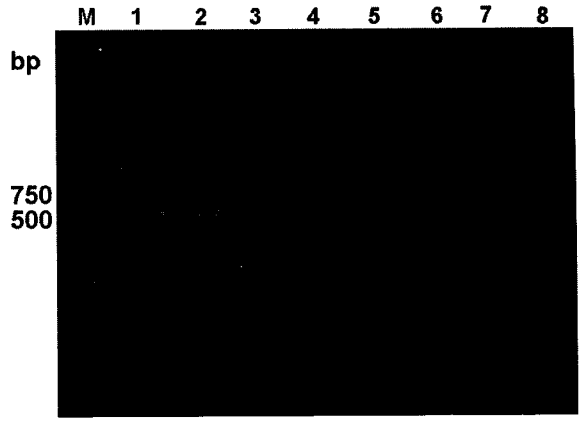

Fig. 5. Amplification products of various amount of the genomic DNA of *Plasmodiophora brassicae*. M: I kb ladder, 1: 10 μg, 2: 1 μg, 3: 100 ng, 4: 10 ng, 5: 1 ng, 6: 100 pg, 7: 10 pg, 8: 1 pg.

resting spores. It was reported that Yano *et al.* (11) successfully disrupted the resting spores of *P. brassicae* with glass beads. In this study, we extracted DNA from resting spores by grinding freeze-dried resting spores or infected tissue in liquid nitrogen. We found that this method was most effective to break resting spores. Our results suggested that PCR-based detection method for *P. brassicae* has potential use for diagnosis and forecasting of clubroot. Attempts to detect resting spores of *P. brassicae* in soil by PCR is in progress.

요 약

십자화과의 뿌리혹병을 발생시키는 *Plasmodiophora brassicae*를 검출하기 위하여 PCR을 이용한 방법을 사용하였다. DNA 염기배열정보를 바탕으로 ribosomal DNA의 small subunit(18S-like)와 ITS 부위로부터 *P. brassicae*에 대한 특이 primer를 작성하여 사용하였다. Primer ITS 5와 PB-C는 *P. brassicae* DNA의 PCR에서 520 bp 크기의 단편을 증폭하였으며, 토양에서 많이 발견되는 균류와 두 종의 다른 균류의 DNA는 증폭하지 않았다. 작성된 Primer를 사용하여 병든 뿌리 조직내의 *P. brassicae*를 검출할 수 있었으며 southern hybridization을 통하여 증폭된 단편이 *P. brassicae*에서 유래된 것임을 확인하였다.

REFERENCES

1. Cenis, J. L. 1992. Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Res.* 20 : 2380.
2. Chen, K. H., Guo, J. R., Wu, X. Y., Loi, N., Carraro, L., Guo, Y. H., Chen, Y. D., Osler, R., Pearson, R. and Chen,

T. A. 1993. Comparison of monoclonal antibodies, DNA probes, and PCR for detection of grapevine yellows disease agent. *Phytopathology* 83 : 915-922.

3. Hawksworth, D. L., Sutton, B. C. and Pegler, D. N. 1995. Ainsworth and Bisby's Dictionary of the Fungi 8th edition. Commonwealth Mycological Institute, Kew.
4. Jarausch, W., Lansac, M., Saillard, C., Broquaire, J. M. and Dosba, F. 1998. PCR assay for specific detection of European stone fruit yellows *Phytoplasmas* and its use for epidemiological studies in France. *Eur. J. of Plant Pathol.* 104 : 17-27
5. Johanson, A. and Jeger, M. J. 1993. Use of PCR for detection of *Mycosphaerella fijiensis* and *M. musicola*, the causal agents of Sigatoka leaf spots in banana and plantain. *Mycol. Res.* 90 : 670-674.
6. Nazar, R. N., Hu, X., Schmidt, J., Culham, D. and Robb, J. 1991. Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of Verticillium wilt pathogens. *Physiol. Mol. Plant Pathol.* 39 : 1-11
7. Rollo, F., Salvi, R. and Torchia, P. 1990. Highly sensitive and fast detection of *Phoma tracheiphila* by polymerase chain reaction. *Appl. Microbiol. Biotechnol.* 32 : 572-576.
8. Rogers, S. O. and Bendich, A. J. 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissue. *Plant Mol. Biol.* 5 : 69-76.
9. Schesser, K., Luder, A. and Henson, J. M. 1991. Use of polymerase chain reaction to detect the take-all fungus, *Gaeumannomyces graminis* in infected wheat plant. *Appl. Environ. Microbiol.* 57 : 553-557.
10. Takahashi, K. and Yamaguchi, T. 1988. A method for assessing the pathogenic activity of resting spores of *Plsamodiophora brassicae* by fluorescence microscopy. *Ann. Phytopath. Soc. Jpn.* 54 : 466-475.
11. Yano, S., Tanaka, S., Ito, S. and Kameya-Iwaki, M. 1997. Variations of random amplified polymorphic DNA (RAPD) patterns among field populations of *Plasmodiophora brassicae. Ann. Phytopath. Soc. Jpn.* 63 : 179-182.
12. Wakeham, A. J. and White, J. G. 1996. Serological detection in soil of *Plasmodiophora brassicae* resting spores. *Physiological and Molecular Plant Pathology* 48 : 289-303.
13. Ward, E. and Adams, M. J. 1998. Analysis of ribosomal DNA sequences of *Polymyxa* species and related fungi and the development of genus- and species-specific PCR primers. *Mycol. Res.* 102 : 965-974.

(Received November 10, 1998)