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Development of an *In Planta* Molecular Marker for the Detection of Chinese Cabbage (*Brassica campestris* ssp. *pekinensis*) Club Root Pathogen *Plasmodiophora brassicae*

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***Plasmodiophora brassicae* is an obligate parasite, a causal organism of clubroot disease in crucifers that can survive in the soil as resting spores for many years. *P. brassicae* causes great losses in susceptible varieties of crucifers throughout the world. In this present study, an *in planta* molecular marker for the detection of *P. bassicae* was developed using an oligonucleotide primer set from the small subunit gene (18S like) and internal transcribed spacer (ITS) region of rDNA. The specific primer sequences determined were TCAGCTTGAATGCTAATGTG (ITS5) and CTACCTCATTTGAGATCTTTGA (PB-2). This primer set was used to specifically detect *P. bassicae in planta*. The amplicon using the specific primer set was about 1,000 bp. However, the test plant and other soil-borne fungi including *Fusarium* spp. and *Rhizoctonia* spp., as well as bacteria such as *Pseudomonas* spp. and *Erwinia* spp. did not show any reaction with the primer set.**

***Key words*:** Chinese cabbage, clubroot, *in planta* detection, *Plasmodiophora brassicae*

The clubroot disease of cruciferous plants including cabbage, Chinese cabbage, radishes, and cauliflower are widely distributed all over the world. Clubroot disease causes great damage in susceptible plant varieties, particularly in Europe and North America. In Korea, this disease was reported for the first time in September of 1920 (17). A disease epidemic has been occuring in Kyunggi and Kangwon Provinces over the last three years in Korea (15). The spread of this disease occurs every year due to the lack of control measures and the mono-cropping of Chinese cabbage (11).

Plasmodiophora brassicae is classified as a member of the Plasmodiophoromycota within the Protozoa Kingdom (12). The resting spores of *P. brassicae* remain in the soil during adverse times or winters (20). These resting spores produce zoospores under favorable temperature and moisture conditions, and the resting spores infect root hairs and produce plasmodium. Plasmodium changes into zoosporangium, and then produces secondary zoospores which infect roots and penetrate into the tissues.

P. brassicae is an obligate endoparasite (4) which is difficult to control. It is also difficult to detect until the symptoms are observed (11). Takahashi and Yamaguchi (21) assessed the pathogenic activity of resting spores using fluorescent microscopy. Wakeham and White (22) developed a serological method to detect the resting spores of *P. brassicae*. However, the detection of the pathogen using microscopic observation has limitations and the serological method is time consuming. Accordingly, the development of a simple and easy method (3, 13) for the detection of this pathogen would be very useful.

In recent years, PCR-based molecular markers have been widely used to detect various plant pathogenic organisms including fungi and bacteria. Plant pathogen detection based on molecular techniques has several advantages compared to other conventional methods. Detection is possible using cultures or *in planta* with equal facilities, even a small qunatity of a pathogen can be detected (5). Rollo *et al.* (18) detected *Phoma tracheiphila* in infected woody tissue using a molecular method. Chen *et al.* (8) also reported that molecular detection of phytoplasma was more sensitive than microscopic or serological methods.

Ribosomal DNA sequence data has been widely used to design pathogen specific molecular primers. Ribosomal DNA are considered to be suitable genomic site for primer design due to their high gene copy number and the nature of the sequences containing both conserved and variable regions. Nazar *et al.* (16) used ribosomal DNA specific primers to detect and differentiate the isolates of

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Verticillium spp. Johanson and Jeger (14) detected *Mycosphaerella fijiensis* and *M. musicola* in banana leaf using primers synthesized from a variable region of the ITS of ribosomal DNA. Recently, a *P. brassicae*-specific primer has been developed which allows for the diagnosis of clubroot disease (7). However, this molecular primer developed by Chee *et al.* (7) is useful only for the detection of isolated resting spores and *in planta* detection has not been verified. Therefore, the objective of this study was to develop a molecular marker for the *in planta* detection and identification of *P. brassicae* based on ribosomal DNA sequence information.

Materials and Methods

Plant material, fungal and bacterial isolates

cv. Daetong and Tambok which are cultivated in alpine areas were used in this study. Chinese cabbages are increasingly being infected with *P. brassicae* nationwide over the past four years. Chinese cabbage roots showing the typical symptoms were colleted from the Daekwangryung area. For the control, healthy plants were grown in a greenhouse. *F. moniliforme*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Phytophthora* sp., *Pseudomonas solanacearum*, and *Erwinia carotovora* were also used for the control.

Genomic DNA preparation

Traditionally, a resting spore suspension of *P. brassicae* was prepared by macerating mature galls in a blender with distilled water and separating spores by filtration through muslin and centrifugation. However, in this study, genomic DNA of *P. brassisae* was isolated from infected plants directly with a modified method of Choi *et al.* (9, 10) and Cenis (6).

PCR amplification of rDNA and PCR-RFLP

The specific primers ITS 1:5'-TCCGTAGGTGAACCTGCGG-3', ITS 4:5'-TCCTCCGCTTATTGATATGCTGC-3, and ITS 5:5'-TCAGCTTGAATGCTAATGTG-3' were used for the amplification of the noncoding region of rDNA, ITSI+5.8S+ITSII (Fig. 1). PCR reactions were performed using a PTC 100 thermocycler (MJ Research, USA). The amplification reactions were performed in 20 μl of 1X reaction buffer, 200 μM dNTPs, 1 unit Taq polymerase, 1.5 mM $MgCl_2$, and 0.5 μM primers. The PCR was programmed for one cycle of 5 minutes at 95°C, followed by 35 cycles of 1 minute at 95°C, 1 minute at 57°C, and 1 minute at 72°C, and one cycle of 10 minutes at 72°C. The reaction products were resolved by electrophoresis on a 1% agarose gel in a 0.5X TBE (0.045M Tris-borate, 0.001M EDTA) buffer for 2 hours and stained with ethidium bromide. Amplified DNA was digested with restriction enzyme *Dde*I. The products were separated in gel containing 2% metaphor agarose (Quantum, USA) and 1% standard agarose, and the DNA was stained with ethidium bromide.

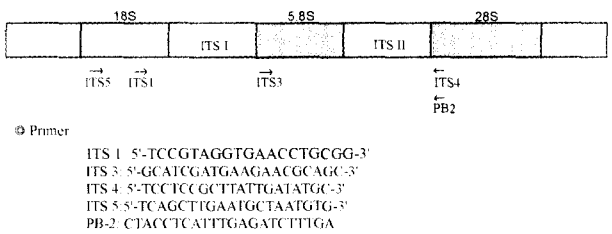

Fig. 1. Genetic map of a portion of the rDNA repeat showing the location of the oligonucleotide primer site used to amplify rDNAs from the *P. brassicae*.

Cloning of ITS regions

Based on the fact that there are differences between the ITS regions of plants and fungi, the genomic DNAs of the infected Chinese cabbage roots, containing both plant and *P. brassicae* DNAs, were amplified at the same time. In order to compare the differences between the ITS regions of the plant and the pathogen, the ITSI+5.8S+ITSII regions were amplified with primers ITS 1 and ITS 4, and 18S+ITSI+5.8S+ITSII regions were amplified with primers ITS 4 and ITS 5. The amplified DNA fragments in the 18S+ITSI+5.8S+ITSII region, which showed the difference in plant and pathogen originated DNAs, were purified with a Wizard PCR Prep (Promega, USA). The cloning of the fragment was then performed with a pGM-T Easy vector (Promega, USA) (19) which was useful for direct ligation (18), and the transformed colonies were selected and cultured for 16 hours in LB broth with ampicillin (50 μg/ml). Finally, the plasmid was extracted with QUANTA-plasmid MiniTM (Quantum, USA).

Primer design and primer selection

The primer sequence was determined by applying the sequencing data from various Chinese cabbage samples to Primer3 (Whitehead Institute/MIT Center for Genome Research, USA), and by the reaction with various fungal and bacterial genomic DNAs.

Southern blotting

Southern blotting and DNA-DNA hybridization were performed according to standard procedures (19). The amplified PCR fragments from the PCR reaction with the primer combination were excised and then labelled using a PCR labelling method (Boehringer Mannheim, Germany). Hybridization was carried out according to the manufacturer's instructions and the methods described by Arrand (1). The signals were detected by exposing the membrane to X-ray film (Agfa CP-BU, Belgium) for 30 minutes using CSPD.

Sensitivity of detection

In order to assess the sensitivity of the molecular detection using the primers, serial ten-fold dilutions of the genomic DNA samples were prepared from the roots of the infected Chinese cabbages. The reactions were prepared with 10 ng, 100 pg, 1 pg, and 0.1 pg of genomic DNA, respectively. The PCR reaction was carried out as described above, and the products were electrophoretically separated on 1.0% agarose gel and stained with ethidium bromide.

Results

PCR amplification of rDNA and PCR-RFLP

The ITSI+5.8S+ITSII regions amplified with the pair primers ITS 1+ITS 4 did not exhibit any differences between the infected and un-infected Chinese cabbage roots. This result indicated that there was no difference between the ITS I+ITS II in the Chinese cabbage and *P. brassicae* (Fig. 2a). However, the amplification products from the reactions with ITS 4+ITS 5 regions showed marked difference between those from the Chinese cabbage and those from the *P. brassicae* (Fig. 2b). In order to differentiate the observed band from *P. brassica*, or from the Chinese cabbage, the band was eluted and treated with a restriction enzyme, *Dde*I. As a result, the detected bands showed a similar molecular weight to *P. brassicae* as determined by Bryan *et al.* (2) and Ward and Adams (23) (Fig. 3).

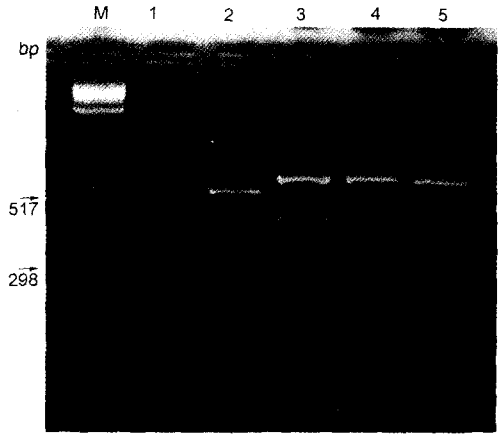

Fig. 3. Restriction fragment length polymorphism of *P. brassicae* and *Brassicae campestris* ssp. *pekinensis* with *Dde*I. M, Molecular marker; 1, uninfected cv. Highland Summer seedling (Control 1); 2, uninfected cv. Highland Summer grown in greenhouse (Control 2); 3, infected cv. Highland Summer grown in the field; 4, infected cv. Daetong grown in the field; 5, infected cv. Tambok grown in the field.

Cloning and sequencing of ITS region

The PCR product was cloned with a pGM-T Easy vector and sequenced using a long Read IR 4200 automatic sequencer (Lincoln, NE. USA). The results of the sequencing are shown in Fig. 4. To identify whether the base sequence obtained from the infected Chinese cabbage matched with that of *P. brassicae*, the sequence data obtained (Fig. 4) was compared with GeneBank data. We confirmed that this data agree with the GeneBank result (data not shown).

Primer design and primer selection

The sequence data (Fig. 4) was found to be that of *P. brassicae*. The primer was then designed based on the nucleotide sequence data using Primer3 (Whitehead Institute/MIT Center for Genome Research, USA). Ward and Adams (23) sequenced the rDNA of several members of Plasmodiophoromycota including *Polymyxa, Spongospora,*

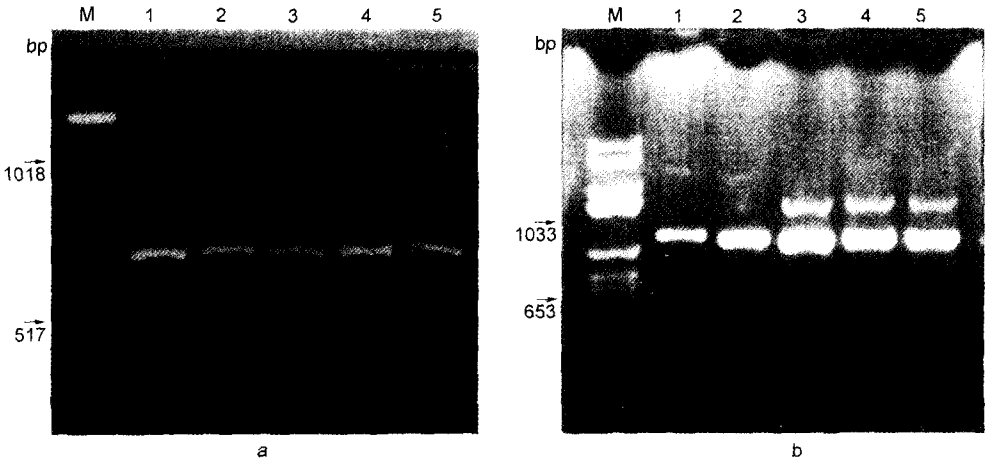

Fig. 2. PCR amplified portion of ITSI+5.8S+ITSII and a part of 18S+ITSI+5.8S+ITSII region in *P. brassicae* (a: primer ITS1+ITS4, b: primer ITS4+ITS5). M, Molecular marker; 1, uninfected cv. Highland Summer seedling (Control 1); 2, uninfected cv. Highland Summer grown in greenhouse (Control 2); 3, infected cv. Highland Summer grown in the field; 4, infected cv. Daetong grown in the field; 5, infected cv. Tambok grown in the field.

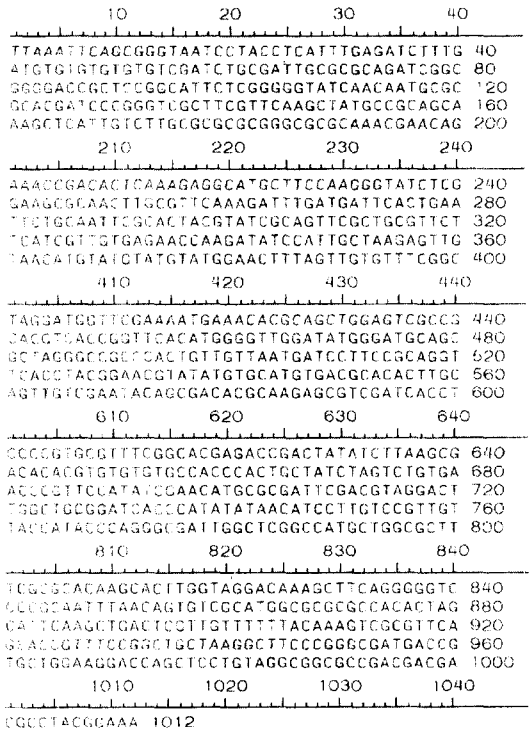

Fig. 4. Complete sequence of a part of the 18S+ITSI+5.8S+ITSII of *P. brassicae* isolated from infected cv. Highland Summer grown in the field.

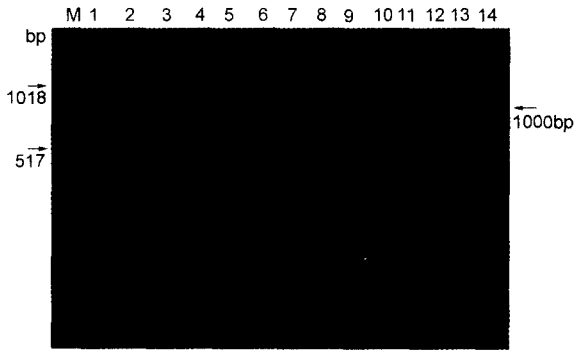

Fig. 5. Amplification of 1,000 bp product using the primerpair ITS5 and PB-2. M, Molecular marker; 1, *Fusarium monilifome* (mating type A+); 2, *Fusarium oxysporum;* 3, *Pseudomonas solanacearum;* 4, *Erwinia carotovora;* 5, *Rhizoctonia solani* (AG-1); 6, *Phytophthora* sp.; 7, uninfected cv. Highland Summer seedling (Control 1); 8, uninfected cv. Highland Summer grown in greenhouse (Control 2); 9, uninfected cv. Highland Summer seedling; 10, uninfected cv. Highland Summer seedling; 11, uninfected cv. Highland Summer seedling; 12, infected cv. Highland Summer grown in the field; 13, infected cv. Daetong grown in the field; 14, infected cv. Tambok grown in the field.

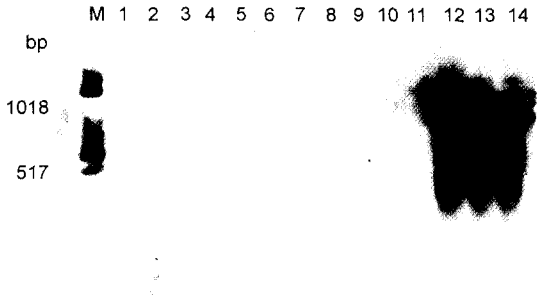

Fig. 6. Result of Southern hybridization with labelled plasmid pGM T-easy vector containing the cloned ITSI+II region from *P. brassicae*. M, Molecular marker; 1, *Fusarium monilifome* (mating type A+); 2, *Fusarium oxysporum;* 3, *Pseudomonas solanacearum;* 4, *Erwinia carotovora;* 5, *Rhizoctonia solani* (AG-1); 6, *Phytophthora* sp.; 7, uninfected cv. Highland Summer seedling (Control 1); 8, uninfected cv. Highland Summer grown in greenhouse (Control 2); 9, uninfected cv. Highland Summer seedling; 10, uninfected cv. Highland Summer seedling; 11, uninfected cv. Highland Summer seedling; 12, infected cv. Highland Summer grown in the field; 13, infected cv. Daetong grown in the field; 14, infected cv. Tambok grown in the field.

Plasmodiophora, Ligniera, and *Olpidium*. Chee *et al.* (7) also designed a primer to detect *P. brassicae* using the genomic DNA of resting spores. However, Chee *et al*'s method was based on the genomic DNA of isolated resting spores, and *in planta* detection was not verified. In this study, however, we designed a specific pair primer to detect *P. brassicae in planta*, and the determined sequence was TCAGCTTGAATGCTAATGTG (ITS5) (GenBank accession number: AF 353997) and CTACCTCATTTGAGATCTTTGA (PB-2) (GenBank accession number: AF 353998). PCR reaction with this pair primer produced a single band (ca. 1,000 bp) specific to *P. brassicae* in the infected Chinese cabbage roots. No amplifications were recorded with *Fusarium* spp., *Rhizoctonia solani*, *Phytophthora* sp., *Erwinia* sp., and healthy Chinese cabbage roots. This result indicated that no corresponding sites of the designed primer were existed in the genomic DNA of *Fusarium* spp., *Rhizoctonia solani*, *Phytophthora* sp., *Pseudomonas* sp., *Erwinia* sp., and healthy Chinese cabbage roots (Fig. 5). Accordingly, the molecular method proposed in this study would seem to be sufficiently sensitive for the *in planta* detection of *P. brassicae* DNA in infected Chinese cabbage root tissues.

Southern blotting

In Southern blotting, which was performed using the ITS region of *P. brassicae*, fragments obtained from the infected Chinese cabbage roots only were found to be hybridized to the PCR products produced from the infected plant roots (Fig. 6). It was revealed from the result that the PCR product from the infected plants was found to be amplified from the genomic DNA of *P. brassicae*. The sensitivity of the detection was highly significant using this method and this might be due to the high copy number of ribosomal genes present in *P. brassicae*.

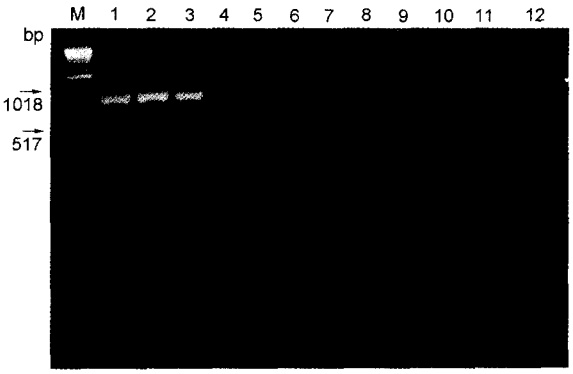

Fig. 7. Amplification products of various amount of the genomic DNA of *P. brassicae*. M, Molecular marker; 1, infected cv. Highland Summer grown in the field (10 ng); 2, infected cv. Daetong grown in the field (10 ng); 3, infected cv. Tambok grown in the field (10 ng); 4, infected cv. Highland Summer grown in the field (100 pg); 5, infected cv. Daetong grown in the field (100 pg); 6, infected cv. Tambok grown in the field (100 pg); 7, infected cv. Highland Summer grown in the field (1 pg); 8, infected cv. Daetong grown in the field (1 pg); 9, infected cv. Tambok grown in the field, (1 pg); 10, infected cv. Highland Summer grown in the field (0.1 pg); 11, infected cv. Daetong grown in the field, (0.1 pg); 12, infected cv. Tambok grown in the field (0.1 pg).

Sensitivity of detection

When serial dilutions of genomic DNA of *P. brassicae* were used as the template DNA, successful amplification was possible up to 1 pg of DNA (Fig. 7). It was also found that the intensity of the bands was in direct proportion to the amount of the template DNA used in the PCR reactions.

Discussion

The plasmodiophorids are a group of obligate, intracellular plant parasites that have been considered as either fungi or protozoa. Plant pathogens in the group include *P. brassicae, Spongospora subterranea* and *P. betae*. Plasmidiophorides have not been cultured free of their host, and there is no base line molecular data available for the group.

Preparation of resting spores of *P. brassicae* from fresh clubroot tissue is laborous. Resting spore suspensions obtained tend to be contaminated by bacteria during storage, even when stored in a refrigerator. Resting spores from some isolated *P. brassicae* showed less stability in the suspension state. In addtion, there is the possibility of cross-contamination between isolates of *P. brassicae* that takes place during the preparation of the resting spores, particularly when different isolates are processed at the same time. In order to overcome these problems, we tried to differentiate the ITS regions of plants and *P. brassicae*.

Recently, ribosomal DNA genes have been widely used for fungal molecular taxonomy, as they consist of a mosaic of regions exhibiting various evolutionary rates. As they are highly conserved, the rDNA sequence can discriminate at the level of orders and kingdoms. At the species level, spacers of the rDNA are widely used for phylogeny studies, as they usually vary between species within a genus, but show little or no intraspecific variation. In addition, the high copy number of the rDNA genes along with the small size of the ITSI+5.8S+ITSII region allow the amplification of very small amounts of DNA (Fig. 7) with PCR. This provides unique opportunities for the detection of minute quantities of pathogen, as is often the case when a fungal pathogen establishes a compatible interaction with its host plant. Hence, its detection in plant samples is complicated. Therefore, in this studies, a rapid PCR-based detection method was developed for detection and identification of *P. brassicae* directly from total DNA extracts of infected plants. This study showed that there was a difference between the ITS regions of plants and fungi. Furthermore, the proposed approaches would be also applicable for the *in planta* detection and genetic analysis of other obligate parasites in plant tissues.

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