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Development of a Rapid Molecular Detection Marker for *Colletotrichum* species with AFLP

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Sweet persimmons have been increasingly cultivated in the southern part of Korea. However, anthracnose disease caused by *Colletotrichum* species is one of the major hindrances in cultivation and productions. In this study, we used polymerase chain reaction (PCR) to detect *Colletotrichum* species with the AFLP (amplified fragment length polymorphism) method. In AFLP, we used E3 (5'-GACTGCGTACCAATTCTA-3') and M1 (5'-GATGAGTCCTGAGTAACAG-3') primer combination and, as a result, 262 bp segment was observed in *Colletotrichum* species only. Specific PCR primers were designed from the sequence data and used to detect the presence of the fungus in genomic DNA isolated from symptomless sweet persimmon plants. Based on sequence data for specific segments, Co.B1 (5'-GAGAGAGTAGAATTGCGCTG-3') and Co.B2 (5'-CTACCATTCTTCTA GGTGGG-3') were designed to detect *Colletotrichum* species. The 220 bp segment was observed in *Colletotrichum* species only, but not in other fungal and bacterial isolates.

KEYWORDS: AFLP, Anthracnose, *Colletotrichum* species, Sweet persimmons

Anthracnose are diseases of the foliage, stems, or fruit that typically appear as dark-colored spots or sunken lesions with a slightly raised rim. Especially, anthracnose diseases of the fruit often result in fruit drop and fruit rot (Agrios, 1998; Vaillancourt and Hanau, 1992). The taxonomy of *Colletotrichum* species has been based on pathogenicity and features such as conidial shape and size, setae and appressorial morphology. However, morphological characteristics are variable in culture, and overlap of phenotypes has made these criteria not always reliable (Agostini *et al.*, 1992). Therefore, early detection of the infection is essential in solving the problem. A method that can provide an early diagnosis of latent infection is needed. Since anthracnose diseases cause serious damage to sweet persimmons and destroys the orchards, it is very important to control the disease in the early stages. However, it is difficult to control the disease unless causal organisms can be isolated from the tissues of sweet persimmons at the early stage of infection, it would be too late to control the disease effectively. Therefore, the key to controlling anthracnose is a quick and precise diagnosis. In recent years, the identification and diagnosis of fungal disease using PCR has became more popular because of its quickness and precision. Molecular markers are becoming an essential tool for gene analysis. In addition to restriction fragment length polymorphic (RFLP), which has been commonly used for linkage analysis, a number of polymerase chain reaction (PCR) based markers such as random amplified polymorphic DNAs (RAPD), sequence tagged sites (STS) and microsatellites have been developed. The PCR-based markers are technically simple and require only a small amount of DNA (Guthrie *et al.*, 1992; Innis and Gelfand, 1990; Mesquita *et al.*, 1998; Mills *et al.*, 1992; Schilling *et al.*, 1996; Sreenivasaprasad *et al.*, 1992; Vakalounakis and Fragkiakakis, 1999; Vilarinhos *et al.*, 1995; William *et al.*, 1990). The use of primers derived from sequences of RAPD markers was first described in plant pathology by Paran and Michelmore (Paran and Michelmore, 1993), who used these sequence characterized amplification regions (SCARs) to identify plant sequences linked to downy mildew resistance genes in lettuce. However, PCR based marker development can be time-consuming if a large number of primers are used. Recently a new technique called amplified fragment length polymorphism (AFLP) was developed by Vos *et al.* (1995). AFLP is a powerful, reliable, stable and rapid assay with potential application in genome mapping, DNA fingerprinting and marker assisted breeding (Old and Primrose, 1994; Reineke and Karlovsky, 2000; Rusell *et al.*, 1997; Sreenivasaprasad *et al.*, 2000). The reproducibility of AFLP is ensured by using restriction site-specific adapters and adapter-specific primers with a variable number of selective nucleotides under stringent amplification conditions. For the efficient control of anthracnose, it is necessary to develop an early diagnosis system, able to detect latent infection of *Colletotrichum* species on sweet persimmons. In this work, we describe the development of PCR primer derived from the AFLP for the specific detection of the *Colletotrichum* spp. in sweet persimmons.

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Table 1. Isolates of *Colletotrichum* species obtained from various locations and used for AFLP

Isolate no.	Isolate name
1	Kyungju 1
2	Kyungju 2
3	Kyungju 3
4	Kyungju 5
5	Kimhae 10
6	Changnyung 17
7	Kimhae 11
8	Kimhae 27
9	Milyang 18
10	Milyang 19
11	Milyang 20
12	Changwon 3
13	Changwon 15
14	Changwon 29
15	Changwon 27
16	Changnyung 16
17	Changnyung

Materials and Methods

Fungal strains and Genomic DNA isolations. The *Colletotrichum* species isolates from the lesion of anthracnose on persimmons in various location were identified as cultural and morphological characters by Chungbuk Univ (Kim *et al.*, 2001; Table 1). Several isolates of *Colletotrichum* species provided from Korean Agricultural Culture Collection (KACC) and Korean Collection for type Cultures (KCTC) were included in the experiment (Table 2). The *Colletotrichum* species isolates were cultivated in PDA (Potato Dextrose Agar) for 7 days at 25°C. The genomic DNA of each isolate was extracted by a modification of the method described by Raeder and Broda (Raeder and Broda, 1985).

AFLP (amplified fragment length polymorphism). The AFLP methods developed by Vos *et al.* were used with some modifications. Genomic DNA (1 μg) was digested with the restriction endonucleases *Eco*RI and *Mse*I. Double-stranded adapters were then ligated to the ends of the restriction fragments followed by ethanol precipitation and resuspension in 40 μl of distilled water. Pre-amplification PCR was performed using standard adapter primers containing no selective nucleotides, followed by selective amplification using similar primers with two or three selective bases (Table 3). Pre-amplification PCR started with a cycle of 30 s at 94°C, 1 min at 60°C, and 1 min at

Table 2. Fungal and bacterial isolates used for the detection of *Colletotrichum* species and for the Southern blot

Isolate no.	Isolate name	*Colletotrichum* species name
1	Kyungju 1	*Colletotrichum* sp.
2	Kyungju 2	*Colletotrichum* sp.
3	Kyungju 3	*Colletotrichum* sp.
4	Kyungju 5	*Colletotrichum* sp.
5	Kimhae 10	*Colletotrichum* sp.
6	Changnyung 17	*Colletotrichum* sp.
7	Kimhae 11	*Colletotrichum* sp.
8	Kimhae 27	*Colletotrichum* sp.
9	Milyang 18	*Colletotrichum* sp.
10	Milyang 19	*Colletotrichum* sp.
11	Milyang 20	*Colletotrichum* sp.
12	Changwon 3	*Colletotrichum* sp.
13	Changwon 15	*Colletotrichum* sp.
14	Changwon 29	*Colletotrichum* sp.
15	Changwon 27	*Colletotrichum* sp.
16	Changnyung 16	*Colletotrichum* sp.
17	Changnyung	*Colletotrichum gloeosporioides*
18	KASS 40641	*C. cricinans*
19	KACC 40700	*C. acutatum*
20	KCTC 6169	*C. gloeosporioides*
21	KACC 40807	*C. higginsianum*
22	KACC 40010	*C. coccodes*
23	C1	*Pestalotiopsis* sp.
24	C2	*Fusarium moniliforme*
25	C3	*Fusarium oxysporum*
26	C4	*Rhizotonia solani*
27	C5	*Pseudomonas* spp.
28	C6	*Plasmodiophora brassicae*
29	C7	*Phytophthora infestans*
30	C8	*Phytophthora capsici*

Table 3. Oligonucleotide adaptors and primers used for AFLP analysis

*Eco*RI-adaptor[a]	CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA
*Mse*I-adaptor[a]	GACGATGAGTCCTGAG TACTCAGGACTCAT
AFLP primer[b]	
*Eco*RI + 0: GACTGCGTACCAATTC	*Mse*I + 0: GATGAGTCCTGAGTAA
*Eco*RI + 2	*Mse*I + 3
E1 GACTGCGTACCAATTC + AT	M1 GATGAGTCCTGAGTAA + CAG
E2 GACTGCGTACCAATTC + AC	M2 GATGAGTCCTGAGTAA + CAC
E3 GACTGCGTACCAATTC + TA	M3 GATGAGTCCTGAGTAA + CTA
E4 GACTGCGTACCAATTC + TG	M4 GATGAGTCCTGAGTAA + CTT

[a] *Eco*RI and *Mse*I adaptors were ligated onto the ends of restriction fragments of template genomic DNAs.
[b] *Eco*RI + 0 and *Mse*I + 0 primers were used in the preamplification of template DNA.

72°C and was followed by 20 cycles. After the pre-amplification, the reaction mixture was diluted to 200 μl with distilled water. For the selective amplification of a limited number of DNA restriction fragments, the secondary template DNA was amplified with primers containing two or three selective 3' nucleotides (*Eco*RI + 2 and *Mse*I + 3 primers). For the selective amplification, the following PCR profile was used: the first cycle of 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C followed by 11 cycles with a stepwise lowering of annealing temperature by 1°C in each cycle and 23 cycles with an annealing temperature of 56°C. Amplification products were separated using standard 6% denatured polyacylamide gel-electrophoresis and detected using autoradiographic procedures.

Development of specific PCR. Selected amplification products were cloned into pGEM®-T Easy Vector (Promega, USA) and transferred into competent *E. coli* according to the supplier's information. Selected clones were sequenced using a silver Sequence™DNA sequencing system (Promega, USA). Based on the determined sequences of a specific fragment of *Colletotrichum* species, the forward and reverse 20-mer primer sets that amplify a single fragment, which can be easily used to screen *Colletotrichum* species Primers were selected with a theoretical melting temperature of 54°C. One primer pair, Co.B1 (5'-GAGAGAGTAGAATTGCGCTG-3') and Co.B2 (5'-CTACCATTCTTCTAGGTG GG-3'), was generated for the detection of *Colletotrichum* species only. PCR reactions were performed in Perkin-Elmer Thermal Cyclers® (USA). The reaction mixtures consisted of 25 ng of genomic DNA, 0.5 μM of each primer, 200 μM dNTP, 1 unit Taq polymerase (Quantum, USA), and 1 × buffer. For the specific amplification, the following PCR profile was used: 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C for 30 cycles. The specificity of the primer pair was tested against *Colletotrichum* species and other fungal and bacterial isolates. Furthermore, different amounts of genomic DNA ranging from 1 pg~100 ng were then prepared by continual dilutions (Innis and Gelfand, 1990).

Dot-hybridization. Standard molecular biological methods were used (Sambook *et al.*, 1996) unless otherwise stated. The specific AFLP DNA fragment was labelled using DIG-11-dUTP (digoxigenein-3-O-methylcarbornyl-(-amino-caproy1-5-(-3-aminoallyl)-uridine-5'-triphosphate) according to the manufacturers' instructions (Specific PCR Reaction, Boehringer-Mannheim, Germany). A total of 100 μl of the PCR labeling reaction mixture containing 10 ng of template DNA, 0.5 μM of each primer (PUC/M13 forward and PUC/M13 reverse), 1 × PCR buffer, 5 units of *Taq* polymerase (Quantum, USA), 0.1 mM of dGTP, dCTP and dATP, 0.09 mM dTTP and 1 mM dUTP was prepared and reacted in a program of 90 s at 94°C, 90 s at 50°C, 2 min at 72°C for 24 cycles and 4 min at 72°C. PCR products amplified by designed primer set (Co.B1 and Co.B2) were electrophoretically separated on 0.8% agarose gel. They were transferred onto Hybond N membranes (Quantum, USA) using a vacuum transfer system. Identification of homologous sequences of DNA, using a labelled probe was possible with the DIG-detection chemiluminescent method (Boehringer-Mannheim).

Results and Discussion

Sequencing of the 262 bp AFLP marker. Different isolates of *Colletotrichum* species were used for AFLP. A total of sixteen primer combinations as shown in Table 2 were screened to identify *Colletotrichum* species. From among the primer combinations, we selected specific bands generated with E3/M1 combination. This primer set is amplified as a 262 bp fragment which was specific to *Colletotrichum* spp. (Fig. 1). The AFLP fragment was

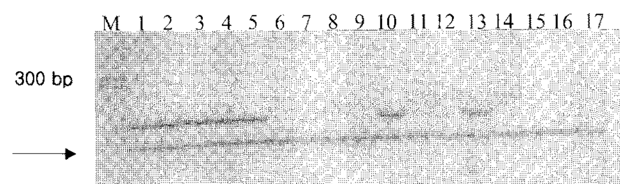

Fig. 1. AFLP profile with *Colletotrichum* spp. using primer combination E3 + M1. Arrow indicates genomic DNA fragment: derived from *Colletotrichum* species. Numbers on the top of the lanes are listed in Table 1.

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  1 CGCATGCTCC CGGCCGCCAT GGCGGCCGCG GGAATTCGAT TGACTGCGTA CCAATTCTAG
 61 AAAGAGAGAG TAGAATTGCG CTGGAGAGGA GTAGGGAACT GCACACTAAA CGAGGGACGG
121 TTGGTGAAGC TTTATACAGG ACTCAAAGTG GCATGTCAGA CGGAGGTGGA GTTGAAGTCG
181 AGAAAAGGTT GGGGGCGGCA AAGACAGGGC CAAGCCGCCA ACTGGTATGA TCTGCAGTCG
241 TGAGCCTCTG GAACCAGTGC CACCCCACCT AGAAGAATGG TAGCTGTTAC TCAGGACTCA
301 TGAATCACTA GTGAATTCGC GGCCGCCTGC AGGTCGACCA TATGGGAGAG CTCCCAACGC
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Fig. 2. Sequence of plasmid insert DNA. The positions of the primers E3 and M1 sequence are underlined and the specific primers Co.B1 and Co.B2 (complimentary) are shaded.

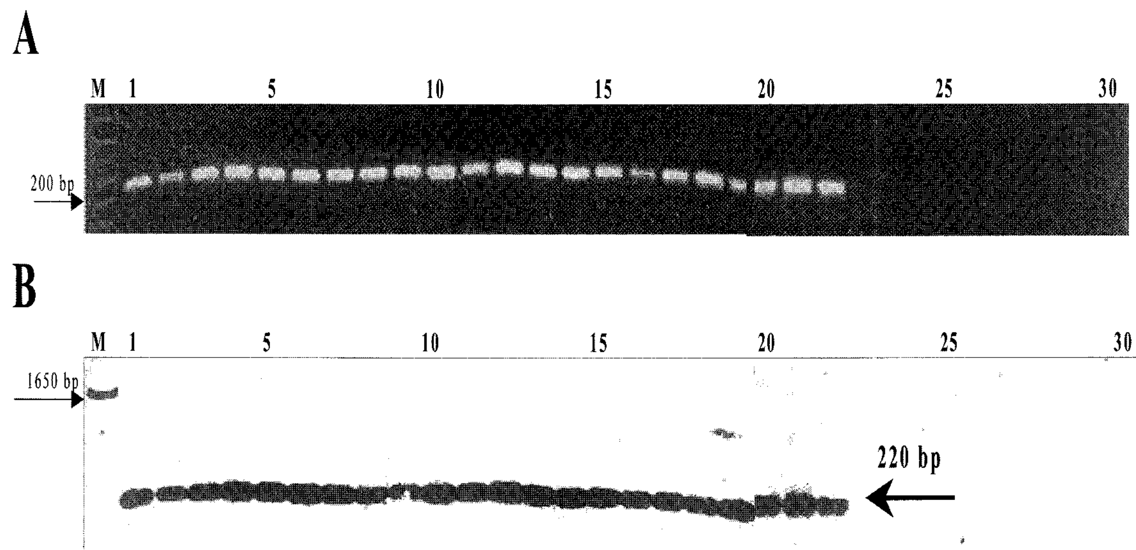

Fig. 3. **A**: The amplification of 220 bp product using primers Co.B1 and Co.B2 to detect *Colletotrichum* spp. **B**: Result of Southern hybridization with a labeled plasmid pGEM®T-easy vector containing the cloned specific band from *Colletotrichum* spp. isolate (M 1 kb DNA ladder; lanes 1~30 are listed in Table 2).

amplified, cloned and sequenced (Fig. 2). The data obtained showed the presence of the initial AFLP primers used (E3 and M1) at both ends of the insert DNA. Primer pair Co.B1 (5'-GAGAGAGTAGAATTGCGCTG-3') and Co.B2 (5'-CTACCATTCTTCTAGGTGGG-3') were designed from the sequence of the selected clone and used to amplify DNA from isolates of *Colletotrichum* species (Fig. 2).

Specific PCR and Dot-hybridization. The primers Co.B1 and Co.B2 were used to amplify a 220 bp band from isolate DNA and from other fungal and bacterial DNA. The pair primer amplified the DNA of all 30 isolates of *Colletotrichum* spp. and none of the other fungal and bacterial isolates (Fig. 3A). Southern blotting of the

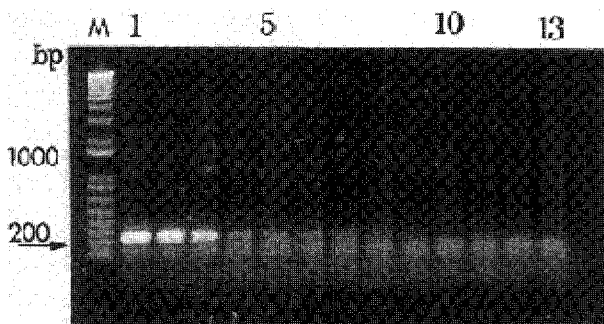

Fig. 4. Primer sensitivity according to template DNA quantities of *Colletotrichum* species by PCR using Co.B1 and Co.B2 primers (M: 1 kb DNA ladder; lane 1, 100 ng; lane 2, 50 ng; lane 3, 10 ng; lane 4, 1 ng; lane 5, 800 pg; lane 6, 600 pg; lane 7, 400 pg; lane 8, 200 pg; lane 9, 100 pg; lane 10, 50 pg; lane 11, 10 pg; lane 12, 5 pg; lane 13, 1 pg).

specific PCR gel and hybridization with labelled insert DNA showed that the PCR products were homologous to the original *Colletotrichum* spp. AFLP marker (Fig. 3B). No signals were observed in lanes of other fungal and bacterial isolates. PCR primer was designed from the sequence of the cloned AFLP marker and used to amplify a 220 bp portion of this fragment.

Sensitivity of the primer set. The specific primer for detection was used to investigate annealing temperature ranges and detectable genomic DNA concentrations. The annealing temperature was a crucial factor in optimizing product formation. Although yields were different, they were detectable in the range of 51°C~58°C on stained agarose gel (data not shown). In addition, when used to elucidate genomic DNA quantity, these primer sets amplified a visible segment at 200 pg~100 ng template DNA levels (Fig. 4).

In this study, sequencing and analysis of AFLP fragment of *Colletotrichum* species allowed us to design specific PCR primer. These primer sets, Co.B1 (5'-GAGAGAGTAGAATTGCGCTG-3') and Co.B2 (5'-CTACCATTCTTCTAGTGGG-3'), successfully amplified DNA fragments from *Colletotrichum* species (Fig. 3). We also investigated the template DNA quantity, since they are factors that could influence the rate and specificity of amplification. In the result, 200 pg~100 ng template DNA levels of *Colletotrichum* species were sufficient for a detectable amplification by PCR. AFLP (amplified fragment length polymorphism) is useful in identifying variation and pathotypes in a wide variety of fungi. Since AFLP will quickly and precisely detect *Colletotrichum* species among the various microfloras that are isolated

from sweet persimmon, then anthracnose infection can be diagnosed before any symptom appears on the tree. Therefore, the development of the primers and probe for identification of *Colletotrichum* species is expected to greatly contribute to the early detection and control of anthracnose disease in sweet persimmons.

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