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PCR-Based Sensitive Detection and Identification of *Xanthomonas oryzae* pv. *oryzae*

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A new primer set was developed for the detection and identification of *Xanthomonas oryzae* pv. *oryzae*, the bacterial leaf blight (BLB) pathogen in rice plant. The nucleotide sequence of *hpaA* gene was determined from *X. o.* pv. *oryzae* str. KACC10331, and the sequence information was used to design primers for the application of the polymerase chain reaction (PCR). The nucleotide sequence of *hpaA* from *X. o.* pv. *oryzae* str. KACC10331 was aligned with those of *X. campestris* pv. *vesicatoria*, *X. campestris* pv. *campestris*, *X. axonopodis* pv. *citri*, and *X. axonopodis* pv. *glycines*. Based on these results, a primer set(XOF and XOR) was designed for the specific detection of *hpaA* in *X. o.* pv. *oryzae*. The length of PCR products amplified using the primer set was 534-bp. The PCR product was detected from only *X. o.* pv. *oryzae* among other *Xanthomonas* strains and reference bacteria. This product was used to confirm the conservation of *hpaA* among *Xanthomonas* strains by Southern-blotting. Furthermore, PCR amplification with XOF and XOR was used to detect the pathogen in an artificially infected leaf. The sensitivity of PCR detection in the pure culture suspension was also determined. This PCR-based detection methods will be a useful method for the detection and identification of *X. o.* pv. *oryzae* as well as disease forecasting.

Key words: *hpaA*, polymerase chain reaction, primer, *Xanthomonas oryzae* pv. *oryzae*

Bacterial leaf blight (BLB) of rice, caused by *X. o.* pv. *oryzae*, is the major disease in all rice growing countries in the tropics and subtropics of Asia [20]. The disease commonly produces leaf blight symptom and the pathogen invades the host tissue through the hydathode on the leaf or through mechanical injuries of the leaf blades and multiple injuries in the vascular systems. Yield losses of 10-20% are common, and losses of 50-70% have been recorded in severely infected fields [20, 21, 26]. Some bactericides have been used to control the disease but none is highly effective and economical. Host plant resistance was found to be the most effective, economical, and environmentally benign method for the control of BLB. So far, 14 dominant (*Xa1, Xa2, Xa3, Xa4, Xa7, Xa10, Xa11, Xa12, Xa14, Xa16, Xa17, Xa18, Xa21*, and *Xa22*) and six recessive(*xa5, xa8, xa13, xa15, xa19*, and *xa20*) resistance genes for BLB have been identified [13, 15, 18]. However, the evolution of new pathogenic races to overcome resistance is always a potential threat to rice production [21].

Isolation of *X. o.* pv. *oryzae* from rice plant and seed by conventional technique is often difficult, usually due to the masking effect of fast-growing, yellow-pigmented bacteria. Biochemical tests [34], serological assays [2], fatty acids, and metabolic profiling [5, 12] have been used in the identification of the pathogen. These assays, however, have limitations such as lack of sensitivity and specificity. Due to problems encountered in conventional methods, polymerase chain reaction (PCR) technology has found a wide application in the detection of plant pathogenic bacteria [3, 8, 19, 25, 30, 31].

PCR allows rapid, specific, and sensitive detection and identification of many plant pathogens. This report

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discusses the development of a PCR-based assay for *X. o.* pv. *oryzae* based on the DNA sequence of the *hpaA* gene.

Materials and Methods

Bacterial strains and culture conditions

Bacterial and fungal strains were obtained from the Korean Agricultural Culture Collection (KACC) in Korea, the Belgian Co-ordinated Collections of Micro-organisms (BCCM^{TM}) in Belgium, and The Ministry of Agriculture, Forest and Fisheries (MAFF) in Japan. All microorganisms used in this study are listed in Table 1. *Xanthomonas* strains were cultured on YDC agar medium (1.0% yeast extract, 2.0% D-[+]-glucose, 2.0% $CaCO_3$, 1.5% agar), *Fusarium* spp. on potato dextrose agar(PDA, Difco) at 28°C for two days, *Escherichia coli* on LB agar [28], and other bacteria on nutrient agar (NA, Difco) at 37°C for a day.

Genomic DNA preparation

Xanthomonas strains were cultured on YDC medium and harvested with a scraper for chromosomal DNA extraction. Total DNA from *Xanthomonas* strains was prepared with the use of the CTAB method as described by Lazo *et al.* [16, 17]. Total DNA from other microorganisms was extracted using the genomic DNA extraction kit [Genomic-tips] supplied by Qiagen (Hilden, Germany).

Sequence alignment and Primer design

The *hpaA* nucleotide sequences of *Xanthomonas* species (*X. campestris pv. campestris, X. axonopodis* pv. *citri, X. oryzae* pv. *oryzae, X. campestri* pv. *vesicatoria, and X. campestris* pv. *glycine*) [6, 14, 24] were aligned by Clustal-method using the DNASTAR software package (DNASTAR Inc.). This software program was used to compare nucleotide sequence and design the primer set from the specific region of *hpaA* sequences of *X. oryzae* pv. *oryzae*(non-conserved regions among other xanthomonads *hpaA* sequences). Each *hpaA* sequence was searched from the GenBank databases of the National Center for Biotechnology Information (NCBI).

PCR amplification

PCR amplifications were carried out with a PTC-225^{TM} thermocycler (MJ Research, Watertown, Mass. USA). PCR reactions were performed in a 50 μL PCR mixture containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM of each dNTP, 10 pM of each primer, and 2 units of *Taq* polymerase (Promega, Madison, Wis. USA). The total amount of genomic DNA from various microorganisms added to the PCR mixture was approximately 50 ng. Reactions were run for 25 cycles, each consisting of 15 sec at 94°C, 15 sec at 58°C, and 30 sec at 72°C, with initial denaturation of 10 min at 94°C and final extension of 7 min at 72°C. An 8-microliter aliquot of each amplified PCR product was electrophoresed on a 1.0% agarose gel, stained with ethidium bromide, and visualized under a UV transilluminator.

DNA dot-blot

DNA dot-blot analysis was carried out to confirm whether the *hpaA* gene was present in other microorganisms including xanthomonads used in this study. A 20-ng sample of genomic DNA isolated from *Xanthomonas* strains and other reference microorganisms was spotted onto Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, UK). These were UV cross-linked to bind the labeled probe DNA. PCR product from *X. o.* pv. *oryzae* str. KACC10331 was labeled as probe with [α-^{32}P]dCTP using the random primed method according to the manufacturer's instructions (Ladderman^{TM} Labeling kit, Takara, Japan). Prehybridization and hybridization were conducted in hybridization buffer (0.75 M NaCl, 75 mM sodium citrate, 0.5% SDS, 0.1% BSA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 50 g/ml denatured salmon sperm DNA) at 65°C for 18 h. After hybridization, the filter was washed twice 10 min each) in 2×SSC containing 0.1% SDS at room temperature and twice (15 min each) in 0.1×SSC containing 0.1% SDS at 65°C. Autoradiography was done at -70°C with CURIX X-ray film (AGFA, Belgium).

PCR detection of pathogen in infected rice

To detect *X. o.* pv. *oryzae*, artificially infected leaves (inoculation of pathogen by pin-point method using toothpick) and healthy rice leaves were obtained from the fields of Honam Agricultural Research Institute (HARI) in Jeonbuk, Korea. One gram of each infected and healthy leaves were sampled with sterile scissors and total DNA was directly extracted from the sample by the CTAB method [23]. Isolated DNA from plants was used in PCR assays as described above.

Table 1. List of bacterial and fungal strains used in this study.

Bacterial and fungal isolate	Source*	Geographical origin
Xanthomonas oryzae pv. *oryzae* (KXO85)	KACC10331 (K1 race)	Korea
Xanthomonas oryzae pv. *oryzae* (KXO595)	KACC10878 (K1 race)	Korea
Xanthomonas oryzae pv. *oryzae*	KACC10379 (K1 race)	Korea
Xanthomonas oryzae pv. *oryzae*	KACC10381 (K2 race)	Korea
Xanthomonas oryzae pv. *oryzae*	KACC10383 (K2 race)	Korea
Xanthomonas oryzae pv. *oryzae*	KACC10384 (K3 race)	Korea
Xanthomonas oryzae pv. *oryzae*	KACC10385 (K3 race)	Korea
Xanthomonas oryzae pv. *oryzae* (PXO99A)	KACC10883	Philippines
Xanthomonas oryzae pv. *oryzae* (PXO99)	KACC10884	Philippines
Xanthomonas oryzae pv. *oryzae* (PXO86)	KACC10885	Philippines
Xanthomonas oryzae pv. *oryzae*	MAFF311018[e]	Japan
Xanthomonas oryzae pv. *oryzae*	MAFF311019	Japan
Xanthomonas oryzae pv. *oryzae*	MAFF311020	Japan
Xanthomonas campestris pv. *campestris*	KACC10913 (ATCC 33913)	United Kingdom
Xanthomonas campestris pv. *campestris*	Chungnam Univ.	Korea
Xanthomonas sesami	KACC10648	Belgium
Xanthomonas translucens vauterin	KACC10674	Belgium
Xanthomonas codiaei vauterin	KACC10909	Tonga
Xanthomonas campestris pv. *carotae*	KACC10164 (ATCC10547)	USA
Xanthomonas campestris pv. *glycines*	KACC10445 (LMG7403)	Zambia
Xanthomonas campestris pv. *glycines*	KACC10446	Zimbabwe
Xanthomonas campestris (Pammel 1895) Dowson	KACC10490	
Xanthomonas campestris pv. *aurantifolii*	KACC10161	
Xanthomonas axonopodis pv. *citri*	KACC10443	Korea
Xanthomonas axonopodis pv. *citri*	KACC10444	Korea
Xanthomonas axonopodis pv. *axonopodis*	KACC10935	Columbia
Xanthomonas fragariae	KACC11115(DSMZ3587)	USA
Xanthomonas campestris pv. *malvacearum*	KACC11127(DSMZ1220)	Germany
Xanthomonas campestris pv. *pelargonii*	KACC11128(DSMZ50857)	Germany
Xanthomonas campestris pv. *juglandis*	DSM1049	United Kingdom
Xanthomonas arboricola pv. *poinsettiicola*	LMG 5403	New Zealand
Xanthomonas axonopodis pv. *axonopodis*	LMG 538	Columbia
Xanthomonas axonopodis pv. *begoniae*	LMG 551	United Kingdom
Xanthomonas axonopodis pv. *dieffenbachiae*	LMG 695	Brazil
Xanthomonas axonopodis pv. *malvacearum*	LMG 761	Sudan
Xanthomonas axonopodis pv. *phaseoli*	LMG 7455	Vulgaria
Xanthomonas axonopodis pv. *phyllanthi*	LMG 844	Sudan
Xanthomonas axonopodis pv. *vasculorum*	LMG 901	Mauritius
Xanthomonas cempestris pv. *vesicatoria*	LMG 905	
Xanthomonas cassavae	LMG 673	Malawi
Xanthomonas cucurbitae	LMG 8662	New Zealand
Xanthomonas pisi	LMG 847	Japan
Xanthomonas theicola	LMG 8684	Japan
Xanthomonas translucens pv. *cerealis*	LMG 679	USA
Xanthomonas translucens pv. *hordei*	LMG 882	Canada
Xanthomonas translucens pv.*phleipratensis*	LMG 843	USA
Pseudomonas fluorescens	KACC10327 (LMG 1794)	United Kingdom
Pseudomonas stutzeri	KACC10290 (ATCC 17588)	
Pectobacterium carotovorum subsp. *carotovorum*	KACC10436 (LMG 2435)	Italy
Pectobacterium carotovorum subsp. *carotovorum*	KACC10440 (LMG 2433)	The Netherlands
Fusarium oxysporum f. sp. *dianthi*	KACC40529 (ATCC11939)	
Fusarium oxysporum f. sp. *cucumerinum*	KACC40525 (ATCC16016)	Germany
Escherichia coli (DH10B)	KACC10765 (ATCC 35150)	

* **KACC**, Korean Agricultural Culture Collection, Korea (http://mgd.niast.go.kr); **ATCC**, American Type Culture Collection, USA; **LMG**, The Belgian Co-ordinated Collections of Micro-organisms (BCCM^{TM}), Belgium; **DSMZ**, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany; **MAFF**, The Ministry of Agriculture, Forest and Fisheries, Japan.

Sensitivity of PCR detection

To evaluate the sensitivity of PCR, 1 g of symptomless regions (over 2 cm distance from lesions) of inoculated rice leaves were sampled, and DNA was extracted and tested in PCR. Furthermore, the sensitivity of PCR detection in the pure culture suspension was determined with a serial dilution of *X. o.* pv. *oryzae* str. KACC10331 cells. A suspension of 5 OD_{600} was diluted six times, first in five-fold and next in two-fold series. The 5-microliter aliquots of each dilution were directly used in the PCR for amplification as described above. After the PCR amplification, DNA concentration of the PCR amplicons was measured by UV spectrophotometer(BECKMAN COUTLER™ DU-650 series, USA).

Nucleotide sequence accession number

The nucleotide sequences presented in this paper were from the GenBank sequence databases under accession numbers AE012221 (*X. campestris* pv. *campestris*), AE011666 (*X. axonopodis* pv. *citri*), AF056246 (*X. campestri* pv. *vesicatoria*), and AF499777 (*X. campestris* pv. *glycines*). *hpaA* nucleotide sequence of *X. o.* pv. *oryzae* was determined from the *X. o.* pv. *oryzae* str. KACC10331 genome project in the laboratory.

Results

Sequence similarity of *hpaA* genes

The nucleotide sequence of *hpaA* genes of *Xanthomonas* strains (*X. o.* pv. *oryzae*, *X. c.* pv. *vesicatoria*, *X. c.* pv. *campestris*, *X. c.* pv. *glycines*, and *X. a.* pv. *citri*) was aligned and analyzed by Clustal-method using the DNA star Meg-align program (Fig. 1). Results confirmed that the *hpaA* genes among *Xanthomonas* strains were conserved and shared substantial homology. *X. o.* pv. *oryzae* has 91.5% identity to *X. c.* pv. *vesicatoria*, 62.7% identity to *X. c.* pv. *campestris*, and 79.5% identity to *X. c.* pv. *glycines* and *X. a.* pv. *citri*. Particularly, the highest homology (99% identity) was observed between *X. c.* pv. *glycines* and *X. a.* pv. *citri* (Fig. 1a). Based on the nucleotide sequence alignment data, genetic distance was used to construct a phylogenetic tree of *hpaA* genes (Fig. 1b). The phylogenetic tree showed a high genetic similarity of sequence pair distance between *X. c.* pv. *glycines* and *X. a.* pv. *citri*. In any case, the phylogenetic tree of *hpaA* genes among *Xanthomonas* strains showed high genetic similarities

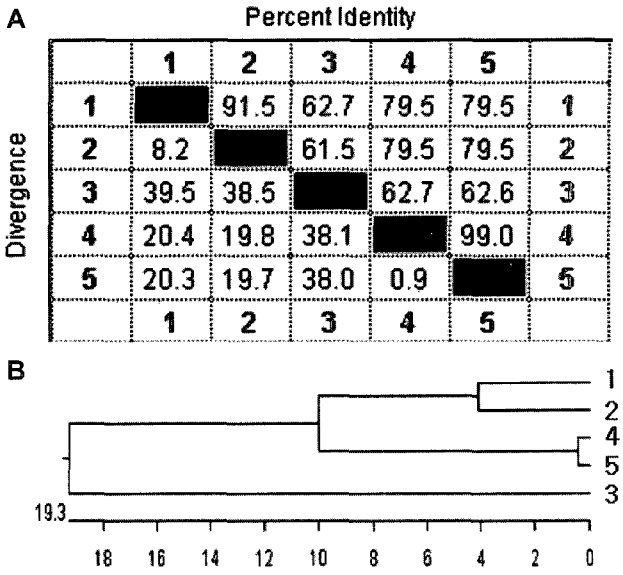

Fig. 1. (A), (B). Genetic relationship analysis of *hpaA* gene from *Xanthomonas* strains. A) Sequence distances of *Xanthomonas* strains. Divergence was calculated by comparing sequence pairs in relation to the phylogeny. Percent identity was directly compared with amino acid sequences, without accounting for phylogenetic relationships. B) Phylogenetic tree. The units at the bottom of the tree indicate the number of substitution events. 1: *X. o.* pv. *oryzae* str. KACC10331, 2: *Xanthomonas campestris* pv. *vesicatoria*, 3: *Xanthomonas campestris* pv. *campestris*, 4: *Xanthomonas campestris* pv. *glycines*, 5: *Xanthomonas axonopodis* pv. *citri*

ranging from 0.4 to 19.3 (substitution events).

PCR amplification

The 20-mer oligonucleotides XOF(5'-ATGCCGATCAC CATGCCGAT-3') and XOR(5'-TGGCCTTGTCGTAC GAGCTC-3') were designed and tested for *X. o.* pv. *oryzae* (Fig. 2). As expected, a 534-bp DNA fragment was amplified with *X. o.* pv. *oryzae*. To check the specificity of the primers, a large collection of other microorganisms (53 microbes), including *Xanthomonas* species and their pathovars, was tested in PCR assay with primers XOF and XOR. None of the other *Xanthomonas* strains and reference microorganisms reacted with the primers (lane 14-53); only *X. o.* pv. *oryzae* (lane 1-13) showed a single amplified DNA fragment (Fig. 3). The primer pair, XOF and XOR, amplified a 534-bp DNA fragment from *X. o.* pv. *oryzae*, when 50 ng DNA was used as the template under optimized conditions (50 μL PCR reaction, containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pM of each primer, 2 units of *Taq* polymerase, and 5 μL of 10×PCR buffer). Similar conditions yielded reproducible results in the Perkin Elmer 9600 thermal cyclers (Perkin Elmer International,

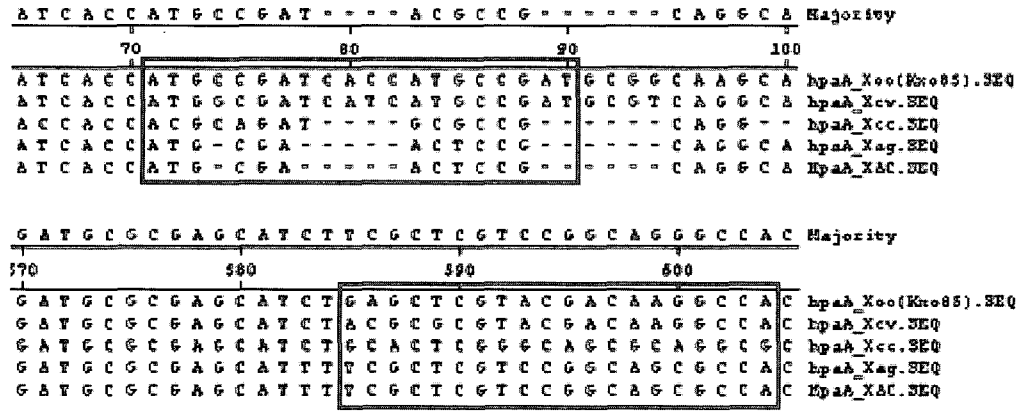

Fig. 2. Nucleotide sequences alignment of the *hpaA gene* and the specific primer design using DNASTAR software package (DNASTAR Inc.). Each strains were denoted on the right. Matched residues were enclosed in the boxes. (*Xac*: *X. axonopodis* pv. *citri*, *Xoo*: *X. oryzae* pv. *oryzae*, *Xcc*: *X. campestris* pv. *campestris*, *Xcv*: *X. campestris* pv. *vesicatoria*, *Xag*: *X. axonopodis* pv. *glycines*). F: Foward primer, R: Reverse primer.

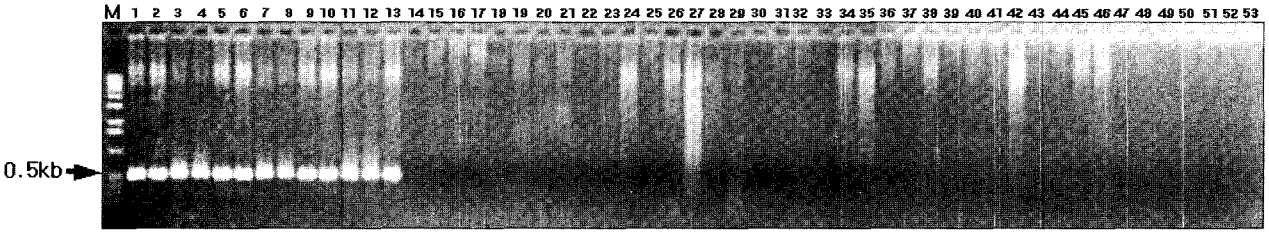

Fig. 3. Specific PCR amplification of partial *hpaA* gene from *X. oryzae* pv. *oryzae* with XOF and XOR. M, Size marker (1 kb DNA ladder; Gibco BRL); lane 1, *X. o.* pv. *oryzae* KXO85; lane 2, *X. o.* pv. *oryzae* KXO595; lane 3, *X. o.* pv. *oryzae* KACC10379; lane 4, *X. o.* pv. *oryzae* KACC10381; lane 5, *X. o.* pv. *oryzae* KACC10383; lane 6, *X. o.* pv. *oryzae* KACC10384; lane 7, *X. o.* pv. *oryzae* KACC10385; lane 8, *X. o.* pv. *oryzae* PXO99A; lane 9, *X. o.* pv. *oryzae* PXO99; lane 10, *X. o.* pv. *oryzae* PXO86; lane11, *X. o.* pv. *oryzae* MAFF311018, lane 12, *X. o.* pv. *oryzae* MAFF311019; lane 13, *X. o.* pv. *oryzae* MAFF311020; lane 14 to 15, *X. c.* pv. *campestris* KACC10913; lane 16, *X. sesami*; lane 17, *X. translucens vauterin*; lane 18, *X. codiaei vauterin*; lane 19, *X. c.* pv. *carotae*; lane 20 to 21, *X. c.* pv. *glycines*; lane 22, *X. campestris* (Pammel 1895) Dowson; lane 23, *X. c.* pv. *aurantifolii*; lane 24 to 25, *X. a.* pv. *citri*; lane 26, *X. a.* pv. *axonopodis* KACC10935; lane 27, *X. fragariae*; lane 28, *X. c.* pv. *malvacearum*; lane 29, *X. c.* pv. *pelargonii*; lane 30, *X. c.* pv. *juglandis*; lane 31, *X. arboricola* pv. *poinsettiicola*; lane 32, *X. a.* pv. *axonopodis* LMG538; lane 33, *X. a.* pv. *begoniae*; lane 34, *X. a.* pv. *dieffenbachiae*; lane 35, *X. a.* pv. *malvacearum*; lane 36, *X. a.* pv. *phaseoli*; lane 37, *X. a.* pv. *phyllanthi*; lane 38, *X. a.* pv. *vasculorum*; lane 39, *X. c.* pv. *vesicatoria*; lane 40, *X. cassavae*; lane 41, *X. cucurbitae*; lane 42, *X. pisi*; lane 43, *X. theicola*; lane 44, *Xanthomonas translucens* pv. *cerealis*; lane 45, *Xanthomonas translucens* pv. *hordei*; lane 46, *Xanthomonas translucens* pv.*phleipratensis*; lane 47, *Pseudomonas fluorescens*; lane 48, *Pseudomonas stutzeri*; lane 49 to 50, *Pectobacterium carotovorum* subsp. *carotovorum*; lane 51, *Fusarium oxysporum* f. sp. *Dianthi*; lane 52, *Fusarium oxysporum* f. sp. *cucumerinum*; lane 53, *Escherichia coli* (DH10B).

Rotkreuz). *Taq* polymerase enzymes supplied by different manufacturers (Promega, Takara, and Toyobo) also yielded similar PCR results.

DNA dot-blot analysis

The presence of the *hpaA* gene in *Xanthomonas* strains and other microorganisms was confirmed by hybridization in a DNA dot-blot. The negative results in hybridization indicated that this gene did not share substantial homology with other bacteria and fungi (lane 8, 20, 22, 30, 33, 47-53) (Fig. 4). In addition, this result suggested that the *hpaA* genes among the microorganisms might be highly conserved.

PCR detection of pathogen

Twelve rice cultivar leaves (susceptible and resistant cultivars) were artificially inoculated and sampled for PCR assays. Healthy leaves were used as control. A 534-bp DNA fragment was amplified from artificially infected rice leaves (B; lane 1-12), but not from healthy leaves (A; lane 1-12) (Fig. 5). Primers did not amplify any fragment of DNA from the various rice plants. In the sensitivity test, the 534-bp DNA fragment was not amplified from the symptomless regions (over 2 cm distance from lesions) of infected resistant rice cultivars (R5: Daechung, R9: Seokwang). Otherwise, symptomless regions of susceptible

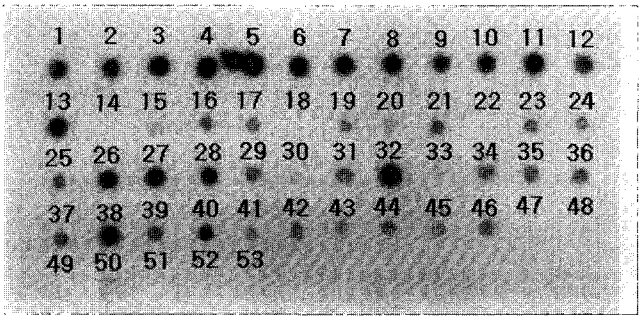

Fig. 4. DNA dot-blot analysis of *hpaA* with PCR-amplified fragment (534-bp) from *X. oryzae* pv. *oryzae* KXO85. 1, *X. o.* pv. *oryzae* KXO85; 2, *X. o.* pv. *oryzae* KXO595; 3, *X. o.* pv. *oryzae* KACC10379; 4, *X. o.* pv. *oryzae* KACC10381; 5, *X. o.* pv. *oryzae* KACC10383; 6, *X. o.* pv. *oryzae* KACC10384; 7, *X. o.* pv. *oryzae* KACC10385; 8, *X. o.* pv. *oryzae* PXO99A; 9, *X. o.* pv. *oryzae* PXO99; 10, *X. o.* pv. *oryzae* PXO86; 11, *X. o.* pv. *oryzae* MAFF311018, 12, *X. o.* pv. *oryzae* MAFF311019; 13, *X. o.* pv. *oryzae* MAFF311020; 14 to 15, *X. c.* pv. *campestris* KACC10913; 16, *X. sesami*; 17, *X. translucens vauterin*; 18, *X. codiaei vauterin*; 19, *X. c.* pv. *carotae*; 20 to 21, *X. c.* pv. *glycines*; 22, *X. campestris* (Pammel 1895) Dowson; 23, *X. c.* pv. *aurantifolii*; 24 to 25, *X. a.* pv. *citri*; 26, *X. a.* pv. *axonopodis* KACC10935; 27, *X. fragariae*; 28, *X. c.* pv. *malvacearum*; 29, *X. c.* pv. *pelargonii*; 30, *X. c.* pv. *juglandis*; 31, *X. arboricola* pv. *poinsettiicola*; 32, *X. a.* pv. *axonopodis* LMG538; 33, *X. a.* pv. *begoniae*; 34, *X. a.* pv. *dieffenbachiae*; 35, *X. a.* pv. *malvacearum*; 36, *X. a.* pv. *phaseoli*; 37, *X. a.* pv. *phyllanthi*; 38, *X. a.* pv. *vasculorum*; 39, *X. c.* pv. *vesicatoria*; 40, *X. cassavae*; 41, *X. cucurbitae*; 42, *X. pisi*; 43, *X. theicola*; 44, *Xanthomonas translucens* pv. *cerealis*; 45, *Xanthomonas translucens* pv. *hordei*; 46, *Xanthomonas translucens* pv.*phleipratensis*; 47, *Pseudomonas fluorescens*; 48, *Pseudomonas stutzeri*; 49 to 50, *Pectobacterium carotovorum* subsp. *carotovorum*; 51, *Fusarium oxysporum* f. sp. *Dianthi*; 52, *Fusarium oxysporum* f. sp. *cucumerinum*; 53, *Escherichia coli* (DH10B).

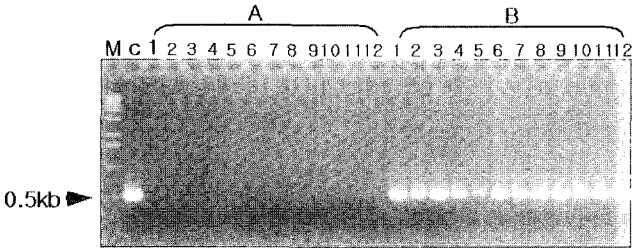

Fig. 5. PCR detection of pathogens in artificially infected rice leaves. A) Healthy rice leaves; B) Infected rice leaves. M, Size marker (1 kb DNA ladder; Gibco BRL); C) *Xanthomonas oryzae* pv. *oryzae* KXO85 (Control); lane 1, Milyang-23; lane 2, Milyang-42; lane 3, Chungchung; lane 4, Samkang; lane 5, Daechung; lane 6, Hangang-chal; lane 7, Pungsan; lane 8, Donjin; lane 9, Seokwang; lane 10, Choongkuk-45; lane 11, Hwayoung; lane 12, Dongjin-1.

rice cultivars were detected up to 12 cm distance from the lesions (R1: Milyang-23, R3: Chungchung) (Fig. 6). It was

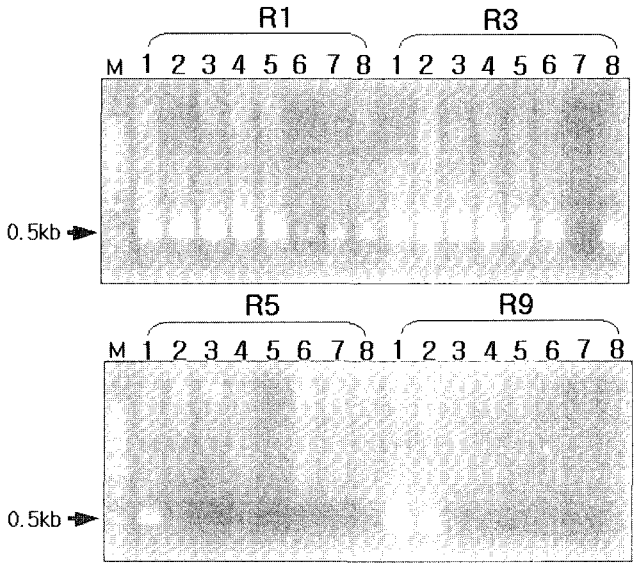

Fig. 6. PCR detection of pathogen in symptomatic or symptomless leaves of inoculated rice cultivars. R1, Milyang-23 (susceptible); R3, Chungchung (susceptible); R5, Daechung (resistance); R9, Seokwang (resistance); M, Size marker (1 kb DNA ladder; Gibco BRL); lane 1, lesions; lane 2, 2 cm distant from lesions; lane 3, 4 cm from lesions; lane 4, 6 cm from lesions; lane 5, 8 cm from lesions; lane 6, 10 cm from lesions; lane 7, 12 cm from lesions; lane 8, 14 cm from lesions.

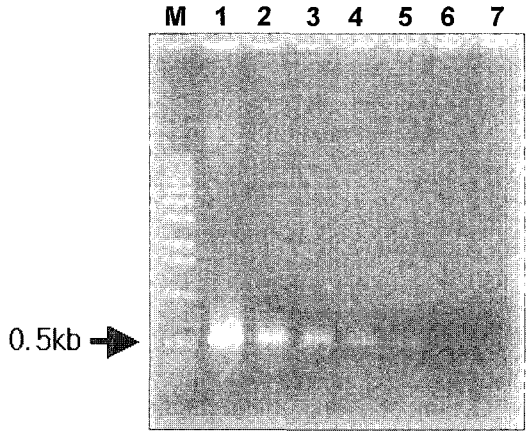

Fig. 7. Amplification of a 534-bp DNA fragment from a dilution series of pure cultured *X. o.* pv. *oryzae* cells. M, Size marke (1 kb DNA ladder; Gibco BRL); lane 1, 5 OD_{600} (9.5×10^4 CFU μL) cells; lane 2, 1 OD_{600} (1.9×10^4 CFU/μL); lane 3, 0.5 OD_{60} (9.5×10^3 CFU/μL); lane 4, 0.25 OD_{600} (4.8×10^3 CFU/μLl); lane 5 0.125 OD_{600} (2.4×10^3 CFU/μL); lane 6, 0.0625 OD_{600} (1.2×10 CFU/μL); lane 7, 0.03125 OD_{600} (6×10^2 CFU/μL).

possible to detect *X. o.* pv. *oryzae* from the pure culture suspensions tested by PCR. The minimum number of cells detected was about 1/16 OD_{600} (1.2×10^3 CFU/μL)and the concentration of amplified DNA fragment in the PCR product was 2.7 ng/μL (Fig. 7).

Discussion

This study developed a new primer set for the detection and identification of *X. o.* pv. *oryzae*. Development of specific DNA probes and primers for detection has been reported for a number of plant pathogenic bacteria [9, 27]. Some of these DNA probes and primers were developed from random screening of cloned fragments or insertion sequences of total genomic DNA [7, 11, 33]. This study focused on the *hpaA* gene for the detection and identification of *X. oryzae* pv. *oryzae* with the use of PCR assay. In most pathogens containing the *hrp* cluster (for hypersensitive reaction and pathogenicity), essential pathogenicity determinants are encoded by the *hrp* genes. The *hpaA* is specifically required for disease development. In addition, *hpaA* was found to contain two functional nuclear localization signals, which are important for the plant-pathogen. Therefore, *hpaA* is an effector protein that may be translocated into the host cell via the *Hrp* secretion pathway [10].

The nucleotide sequences of *hpaA* genes among *Xanthomonas* strains were not the same but were well conserved. Especially, the *hpaA* nucleotide sequence of *X. campestris* pv. *vesicatoria* was very similar to that of *X. o.* pv. *oryzae* (99% identity) (Fig. 1). The nucleotide sequences of XOF and XOR primer regions of *X. o.* pv. *oryzae* were only two and three nucleotides different from those of *X. campestris* pv. *vesicatoria*, respectively (data not shown). The conservation of *hpaA* genes among *Xanthomonas* strains and other microbes was supported by hybridization studies. However, primers (XOF and XOR) used in this study did not amplify DNA from other pathogenic or non-pathogenic bacteria. PCR techniques with the use of these primers can be applied to detect both pathogens and pathovars of *Xanthomonas oryzae*.

PCR conditions such as primers, template, Mg^{2+} [1, 4], thermocyclers, and thermostable polymerase origin [22, 30] have been shown to affect amplification. In this study, all conditions were optimized of an MJ Research PTC-225 thermocycler to avoid such artifacts and to ensure reproducibility of amplification.

A PCR technique was successfully used to detect viable cells of *X. o.* pv. *oryzae* in artificially infected rice leaves. The presence of plant pathogenic bacteria in symptomless plants (latent infection) has been presented [33]. In this report, the pathogen was detected with the use of PCR assays, although the symptom was not found in susceptible rice cultivars. On the other hand, pathogens were not detected from resistant rice cultivars, except from the lesions. This result suggests that the disease does not expand out of the lesions in resistant rice cultivars.

PCR is considered as the most time-consuming, cost-effective, and rapid method for the detection and identification of pathogenic bacteria, although many improved methods (biochemical test, serological assays, fatty acids, and metabolic profiling) have been developed so far. In addition, Disease forecasting, using rapid methods, is important for assessing the health status of rice, because a latent population can lead to serious epidemics. So, the PCR assay using a primer set (XOF and XOR) designed from the sequence of *hpaA* gene will be a useful tool for the detection and identification of *X. o.* pv. *oryzae*.

국문초록

중합효소연쇄 반응에 의한 벼 흰잎마름병균의 특이적 검출

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본 연구는 벼의 세균병 중 치명적인 흰잎마름병을 유발하는 *Xanthomonas oryzae* pv. *oryzae*를 검출할 수 있는 프라이머를 개발하기 위해 실시하였다. *X. o.* pv. *oryzae* str. KACC10331의 *hpaA*유전자 염기서열로부터 흰잎마름병만을 특이적으로 검출할 수 있는 프라이머를 제작하여 중합효소연쇄반응에 사용하였다. 개발된 특이 프라이머는 *X. o.* pv. *oryzae* str. KACC10331과 *X. campestris* pv. *vesicatoria*, *X. campestris* pv. *campestris*, *X. axonopodis* pv. citri 그리고 *X. axonopodis* pv. *glycines*의 *hpaA*유전자 염기서열의 상동성을 비교하여, 그 중 *X. o.* pv. *oryzae*만이 가지는 특이적인 부분을 바탕으로 각각 20-mer인XOF와 XOR를 제작하였다. 제작된 프라이머를 이용하여 중합효소연쇄반응을 실시한 결과 반응 후 생성된 단편의 크기는 534-bp였다. 반응 후 생성된 단편은 Southern hybridization

을 통하여 *Xanthomonas* 균주들의 *hpaA*유전자 존재 여부 및 그 상동성을 비교분석하기 위해 사용하였다. 또한 제작된 프라이머를 이용하여 흰잎마름병에 감염된 벼 잎에서의 검출 여부를 확인하였고 X. o. pv. oryzae의 순수 균주 배양액을 중합효소연쇄반응에 이용하여 검출한계를 검정하였다. 본 연구에서 제작된 프라이머를 사용한 중합효소연쇄반응 방법은 *X. o.* pv. *oryzae*의 검출 뿐만 아니라 흰잎마름병의 발생 예찰에 매우 유용할 것으로 판단 되었다.

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