Sensitive and Specific Detection of *Xanthomonas oryzae* pv. *oryzae* by Real-Time Bio-PCR Using Pathovar-Specific Primers Based on an *rhs* Family Gene

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Abstract

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The present study describes bio-polymerase chain reaction (PCR) assays to detect bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* in rice. Successful control of *X. oryzae* pv. *oryzae* requires a specific and reliable diagnostic tool. However, other *X. oryzae* pathovars are detected by currently available molecular and serological methods. In this study, SYBR Green real-time and conventional PCR primer sets were designed based on an *rhs* family gene of *X. oryzae* pv. *oryzae* KACC10331 because these genes are structurally diverse. The specificity of the primers was evaluated using purified DNA from 11

isolates of two *X. oryzae* pathovars, 21 other *Xanthomonas* species, and 4 other reference phytopathogenic bacteria and fungi. The assay was also able to detect at least two genome equivalents of cloned amplified target DNA using purified DNA. Thus, the SYBR Green real-time PCR-based method can be used for the rapid and specific detection of *X. oryzae* pv. *oryzae* and will potentially simplify and facilitate diagnosis and monitoring of this pathogen and guide plant disease management.

Bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* is the major rice disease in all tropical and subtropical Asian countries. The disease commonly produces leaf blight symptoms, and the pathogen invades the host tissue through leaf hydathodes or through mechanical injuries of the leaf blades and multiplies in the vascular system. Yield losses of 10 to 20% are common, and losses of 50 to 70% have been recorded in severely infected fields (9,10,14). The control of bacterial blight is difficult, and the only practical methods for disease management rely on appropriate culture practices and the use of pathogen-free seed and resistant cultivars. Therefore, the specific detection of this pathogen in seed and plants is essential for effective disease control (16,21). Diagnosis of *X. oryzae* pv. *oryzae* infection is based on isolation of the pathogen, followed by biochemical identification, pathogenicity tests, or serological tests consisting of fatty acid and other metabolic profiling (2,3,5,19).

Currently, molecular assays based on repeated element, siderophore receptor gene, and 16S-23S rDNA spacer region are widely used for the detection of *X. oryzae* pv. *oryzae* strains but there have been critical defects in the diagnosis and identification of *X. oryzae* pv. *oryzae* isolates, in that these assays also detect *X. oryzae* pv. *oryzicola* (1,7,16,21).

Therefore, in this study, a pathovar-specific primer set based on the *rhs* family gene of *X. oryzae* pv. *oryzae* KACC 10331 was designed. It was reported that *Rhs* repertoires (YD repeat proteins) are highly dynamic among enterobacterial genomes, due to repeated gene gains and losses. In contrast, the primary structures of *rhs* genes are evolutionarily conserved, indicating that *rhs* sequence diversity is driven not by rapid mutation but by the rela-

tively slow evolution of novel core-and-tip combinations. However, the function of these proteins is unknown and the conditions under which they are expressed have also been difficult to define (4).

This article describes the design of specific polymerase chain reaction (PCR) primers that distinguish *X. oryzae* pv. *oryzae* from other xanthomonads and the optimization of protocols for detection of *X. oryzae* pv. *oryzae* from plants.

Materials and Methods

Bacterial strains and culture conditions. Bacterial and fungal strains were obtained from the Korean Agricultural Culture Collection (KACC) in the Republic of Korea, the Ministry of Agriculture, Forestry and Fisheries of Japan, the Belgian Coordinated Collections of Micro-organisms, the Deutsch Sammlung von Mikroorganismen und Zellkulturen GmbH in Germany, and the American Type Culture Collection. All microorganisms used in this study are listed in Table 1. *Xanthomonas* strains were cultured on YGC medium (2.0% D-(+)-glucose, 2.0% CaCO₃, 1.0% yeast extract, and 1.5% agar) at 28°C for 2 days, *Escherichia coli* on Luria-Bertani agar (17) at 37°C for 18 h, and other microbes on nutrient agar (Difco Laboratories) or potato dextrose agar (Difco Laboratories) at 26 to 28°C for 1 to 3 days.

DNA extraction. *Xanthomonas* strains were cultured on YGC medium and harvested with a scraper for total DNA extraction, which was prepared as described (6). Total DNA from other microorganisms was prepared using a genomic DNA extraction kit (Genomic-tips; Qiagen, Hilden, Germany).

Primer design and conventional PCR. Two primer sets were designed for an *rhs* family gene of *X. oryzae* pv. *oryzae* KACC10331 (GenBank accession no. NC_006834, gi|58579623:124816-125631), with predicted PCR products of 114 and 290 bp (Table 2). Conventional PCR assays were performed with a PTC-225 thermocycler (MJ Research, Watertown, MA). All amplifications were carried out in a final volume of 50 µl containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.2 mM each dNTP, 10 pM of each primer, 2 units of *Taq* polymerase (Promega Corp., Madison, WI), and approximately 50 ng of

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genomic DNA from a given microorganism. Reactions were run for 25 cycles, each consisting of 60 s at 94°C, 30 s at 56°C (for the XOO290 primer set), and 60 s at 72°C, with an initial denaturation of 5 min at 94°C and a final extension of 10 min at 72°C. A 10-μl aliquot of each amplified PCR product was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and visualized with a UV transilluminator. For the amplification of insertion sequence (IS1113) region, the primers and reaction conditions were described as by Sakthivel et al. (16).

Specificity and sensitivity of the SYBR Green real-time PCR assay. The SYBR Green real-time PCR assay was performed in a

20-μl reaction volume containing iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA). Each PCR reaction contained 10 μl of iQ SYBR Green Supermix and 10 pmol each of XOO114F/R and 5 ng of purified DNA from each sample. Amplification was carried out using the CFX96 real-time PCR system (Bio-Rad Laboratories, Inc.), and the thermal cycling parameters were as follows: 95°C for 3 min; 45 cycles of 95°C for 10 s and 60°C for 20 s; and a melting curve of 65 to 95°C with an increment of 0.5°C. A copy number of the cloned DNA was calculated with the following equation (20): copies/μl = $[6.022 \times 10^{23} \text{ (copy/mol)} \times \text{amount (g)}] / [\text{length (bp)} \times 660 \text{ (g/mol/bp)}]$. For

Table 1. Bacterial and fungal strains used in specificity tests of conventional and real-time polymerase chain reaction assays^a

No.	Bacterial and fungal isolates	Source ^b	Geographic origin	IS1113 ^c	Siderophore ^d	<i>rhs</i> Family gene ^e
1	<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	KACC 10331	Republic of Korea	+	+	+
2	<i>X. oryzae</i> pv. <i>oryzae</i>	KACC 10878	Republic of Korea	–	+	+
3	<i>X. oryzae</i> pv. <i>oryzae</i>	KACC 10381	Republic of Korea	–	+	+
4	<i>X. oryzae</i> pv. <i>oryzae</i>	KACC 10384	Republic of Korea	+	+	+
5	<i>X. oryzae</i> pv. <i>oryzae</i>	KACC 10385	Republic of Korea	–	–	+
6	<i>X. oryzae</i> pv. <i>oryzae</i>	KACC 10883	Philippines	+	–	+
7	<i>X. oryzae</i> pv. <i>oryzae</i>	KACC 10885	Philippines	–	+	+
8	<i>X. oryzae</i> pv. <i>oryzae</i>	MAFF 311018	Japan	+	+	+
9	<i>X. oryzae</i> pv. <i>oryzae</i>	MAFF 311019	Japan	–	–	+
10	<i>X. oryzae</i> pv. <i>oryzicola</i>	LMG 797	Malaysia	–	–	–
11	<i>X. oryzae</i> pv. <i>oryzicola</i>	LMG 657	India	–	+	–
12	<i>X. campestris</i> pv. <i>campestris</i>	ATCC 33913	United Kingdom	–	–	–
13	<i>X. campestris</i> pv. <i>carotae</i>	ATCC 10547	United States	–	–	–
14	<i>X. campestris</i> pv. <i>glycines</i>	LMG 7403	Zambia	–	–	–
15	<i>X. campestris</i> pv. <i>pelargonii</i>	DSMZ 50857	Germany	–	–	–
16	<i>X. campestris</i> pv. <i>juglandis</i>	DSMZ 1049	United Kingdom	–	–	–
17	<i>X. campestris</i> pv. <i>malvacearum</i>	DSMZ 1220	Germany	–	–	–
18	<i>X. axonopodis</i> pv. <i>citri</i>	KACC10443	Republic of Korea	–	–	–
19	<i>X. axonopodis</i> pv. <i>dieffenbachiae</i>	LMG 695	Brazil	–	–	–
20	<i>X. axonopodis</i> pv. <i>vasculorum</i>	LMG 901	Mauritius	–	–	–
21	<i>X. axonopodis</i> pv. <i>begoniae</i>	LMG 551	United Kingdom	–	–	–
22	<i>X. axonopodis</i> pv. <i>phaseoli</i>	LMG 7455	Belgium	–	–	–
23	<i>X. axonopodis</i> pv. <i>phyllanthi</i>	LMG 844	Sudan	–	–	–
24	<i>X. axonopodis</i> pv. <i>aurantifolii</i>	KACC 10161	Not known	–	–	–
25	<i>X. axonopodis</i> pv. <i>vesicatoria</i>	LMG 905	Tonga	–	–	–
26	<i>X. translucens</i> pv. <i>phleipratensis</i>	LMG 843	United States	–	–	–
27	<i>X. translucens</i> pv. <i>cerealis</i>	LMG 679	United States	–	–	–
28	<i>X. translucens</i> pv. <i>hordei</i>	LMG 882	Canada	–	–	–
29	<i>X. arboricola</i> pv. <i>poinsetticola</i>	LMG 5403	New Zealand	–	–	–
30	<i>X. cassavae</i>	LMG 673	Malawi	–	–	–
31	<i>X. cucurbitae</i>	LMG 8662	New Zealand	–	–	–
32	<i>X. theicola</i>	LMG 8684	Japan	–	–	–
33	<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>	LMG 2435	Italy	–	–	–
34	<i>Pseudomonas syringae</i> pv. <i>tomato</i>	LMG 5093	United Kingdom	–	–	–
35	<i>Escherichia coli</i> (O157:H7)	ATCC 35150	Not known	–	–	–
36	<i>Fusarium oxysporum</i> f. sp. <i>dianthi</i>	ATCC 11939	Not known	–	–	–

^a Symbols: + and – indicate species was detected or not detected, respectively.

^b KACC, Korean Agricultural Culture Collection, Republic of Korea (<http://genebank.rda.go.kr>); MAFF, Ministry of Agriculture, Forestry and Fisheries of Japan; LMG, Belgian Coordinated Collections of Microorganisms (BCCM); ATCC, American Type Culture Collection, United States; DSMZ, German Collection of Microorganisms and Cell Cultures.

^c Putative siderophore receptor (22).

^d According to Sakthivel et al. (16).

^e Positions XOO114 and XOO290 correspond to GenBank accession no. NC_006834, gi|58579623:124816-125631.

Table 2. Sequences of the primers and probes used in this study

Primer, probe	Sequences (5'–3')	Annealing temperature (°C)	Amplicon size (bp)	Gene or position	Reference
TXT	GTCAAGCCAAGTGTGTA	56	964	IS1113	16
TXT4R	CGTTCGCGCCACAGTTG	...	...	...	...
PF	GAATATCAGCATCGGCAACAG	68	152	Siderophore receptor ^a	22
PR	TACCGGAGCTGCGCGTT	...	...	...	...
Probe	FAM-CATCGCCTGCTCGGCTACCAGC-TAMRA	...	...	...	...
XOO114F	GATCGCTCGGTGCGAATGTG	60	114	125476–125589 ^b	This study (Real) ^c
XOO114R	GGCTCGCCCCAGTCCGTCGTA	...	...	...	...
XOO290F	GCGCACCGAGTATTCCTA	56	290	125169–125458 ^b	This study (Conv.) ^c
XOO290R	CTTCGCCGGTCCAGATGA	...	...	...	...

^a Putative siderophore receptor.

^b Positions correspond to GenBank accession no. NC_006834, gi|58579623:124816-125631.

^c Real and Conv. = real-time and conventional polymerase chain reaction.

the amplification of putative siderophore receptor, the primers, probe, and reaction conditions were as described by Zhao et al. (21).

DNA dot-blot analysis. A total volume of 10 µl of genomic DNA (approximately 100 ng) was spotted onto a Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, UK) and then UV

Fig. 1. Sampling area of rice leaves (*Oryza sativa* 'Dongjin'). Assays were performed using the leaf-clip method with *Xanthomonas oryzae* pv. *oryzae* KACC10331; dpi = days postinoculation.

cross-linked to bind the labeled probe DNA, where the PCR product from *X. oryzae* pv. *oryzae* KACC10331 with the XOO114 primer set was labeled as a probe with [³²P]dCTP using the random primed method according to the manufacturer's instructions (Lad-derman Labeling kit; Takara, Japan). The prehybridization and hybridization were performed in hybridization buffer (0.75 M NaCl, 75 mM sodium citrate, 0.5% sodium dodecyl sulfate [SDS], 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, and denatured salmon sperm DNA at 50 µl/ml) at 65°C for 18 h. After hybridization, the membrane was performed at room temperature for 2 × 10 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS and at 65°C for 2 × 15 min in 0.1× SSC and 0.1% SDS. Autoradiography was conducted at -70°C with CURIX X-ray film (AGFA, Belgium).

Detection of pathogen by bio-PCR. *X. oryzae* pv. *oryzae* KACC10331 was grown on YGC medium and bacterial cells were adjusted to an optical density at 600 nm (OD₆₀₀) = 0.5. Bacterial cells were inoculated on independent leaves of 3-week-old seedlings (*Oryza sativa* 'Dongjin') grown in a greenhouse by the leaf-clip method and the samples were taken from an area 5 cm from the lesion area shown at 12 days postinoculation (dpi) (Fig. 1). Each sample was dipped in 500 µl of sterile distilled water for 30 min. Water treated with each sample (2 µl) was used in SYBR Green real-time and conventional PCR assays as described above.

Results

Sequence specificity of designed primers. The specificity of the primer sets based on the *rhs* family gene of *X. oryzae* pv. *oryzae* KACC10331 (GenBank accession no. NC_006834, gi|58579623:124816-125631) was tested in silico by a similarity search against the National Center for Biotechnology Information BLAST sequence database (<http://www.ncbi.nlm.nih.gov/>). There were no significant matches with previously determined sequences.

Specificity test of primers. The primer set XOO290F/R was tested by conventional PCR against *X. oryzae* pv. *oryzae*. As expected, a 290-bp DNA fragment was amplified (Fig. 2). To check the specificity of the primers, a large collection of other microorganisms, including other *Xanthomonas* spp. and their pathogens, was tested by SYBR Green real-time PCR with the XOO114F/R primer set. DNA from the other *Xanthomonas* strains and reference microorganisms was not successfully amplified with these primers; only assays with *X. oryzae* pv. *oryzae* yielded amplified products with fluorescence intensity and a single amplified DNA fragment (Table 1).

Standard curves and melting temperature. We used SYBR Green real-time PCR analysis of *X. oryzae* pv. *oryzae* to generate a standard curve by plotting the mean cycle threshold ($n = 3$) versus the logarithmic concentration of genomic DNA, cloned DNA, and density of the cell suspension (range, 5×10^0 to 5×10^{-6} ng/µl, 1.45×10^9 to 1.45×10^3 copies/µl, and 0.1×10^0 to 0.1×10^{-6} OD₆₀₀ unit of cells, respectively; Fig. 3A; Table 3). The assay exhibited a good linear response ($R^2 = 0.994$). The detection limit

Fig. 2. Specific polymerase chain reaction amplification of an *rhs* family gene fragment from *Xanthomonas oryzae* pv. *oryzae* with the primer set XOO290F/R. Lane M, size marker (1-kb DNA plus ladder; Gibco BRL); lanes 1–36 are listed in Table 1.

of the reaction was one cloned copy of the *rhs* family gene of *X. oryzae* pv. *oryzae*. Standard regression analysis of the linear part of the slope gave a coefficient of -3.311 , which yielded a PCR efficiency of 100.4% (Fig. 3B). Analysis of the melting temperature and melting peaks of *X. oryzae* pv. *oryzae* by SYBR Green real-time PCR revealed a reproducible melting temperature of 85°C and specific peaks (Fig. 3C).

DNA dot-blot analysis. The presence of the *rhs* family gene or of homologs in *Xanthomonas* strains was confirmed by dot-blot analysis. All strains of *X. oryzae* pv. *oryzae* hybridized with a PCR probe (XOO114) specific to the *rhs* family gene. This result suggested that the *rhs* family gene is highly conserved in *X. oryzae* pv.

oryzae (Fig. 4). Assays with DNA from strains of other *Xanthomonas* spp. and other phytopathogens showed no hybridizing signal, indicating that the *rhs* family gene does not exist or is not substantially homologous in other bacteria and fungi.

Detection of the pathogen by bio-PCR in infected rice. Each primer set, XOO114F/R and XOO290F/R, could also detect the pathogen by SYBR Green real-time and conventional PCR in infected rice leaves. Amplified products derived from all infected rice leaves showed fluorescence in the SYBR Green real-time PCR assay, and the expected fragment size of approximately 290 bp was amplified by conventional PCR, whereas no band was amplified from healthy rice leaf samples (Fig. 5).

Fig. 3. Specificity, melting peak, and standard curve of the XOO114F/R primer set using SYBR Green real-time polymerase chain reaction (PCR). **A**, Fluorescence intensity as a function of concentration of template. For each assay, a series of 10-fold dilutions of cloned DNA (range, 1.45×10^9 to 1.45×10^3 copies/ μ l) was used as the template for PCR (1–7, sample dilutions; 8, no template control). **B**, Standard curve derived from the amplification plot. **C**, Melting-peak analysis. The negative first derivative of relative fluorescence units ($-d(RFU)/dT$) is plotted as a function of temperature. Amplified product, 85°C. A high peak indicates amplified product and a low peak is the no-template control.

Table 3. Mean cycle threshold (C_T) end-point fluorescence of 10-fold serial dilutions of *Xanthomonas oryzae* pv. *oryzae* cloned DNA, genomic DNA, and cell suspension as determined by the SYBR Green real-time polymerase chain reaction assay

Cloned DNA		Genomic DNA		Cell suspension	
Plasmid copies/ μ l	C_T values	Weight/ μ l	C_T values	Cell density ^a	C_T values
1.45×10^9	18.32 ± 0.02	5 ng	17.45 ± 0.19	0.1×10^0	18.27 ± 0.22
1.45×10^8	21.75 ± 0.22	500 pg	20.55 ± 0.34	0.1×10^{-1}	19.28 ± 0.14
1.45×10^7	25.52 ± 0.56	50 pg	24.15 ± 0.15	0.1×10^{-2}	22.56 ± 0.08
1.45×10^6	29.02 ± 0.14	5 pg	27.54 ± 0.24	0.1×10^{-3}	26.30 ± 0.11
1.45×10^5	32.19 ± 0.25	500 fg	30.95 ± 0.29	0.1×10^{-4}	30.14 ± 0.09
1.45×10^4	35.78 ± 0.59	50 fg	33.91 ± 0.84	0.1×10^{-5}	33.76 ± 0.43
1.45×10^3	37.65 ± 0.03	5 fg	37.82 ± 0.85	0.1×10^{-6}	36.97 ± 0.61

^a Optical density at 600 nm unit of cells.

Fig. 4. DNA dot-blot analysis of the *rhs* family gene with a polymerase chain reaction-amplified fragment (114 bp) from *Xanthomonas oryzae* pv. *oryzae*. Lanes 1–9, *X. oryzae* pv. *oryzae*; lanes 10–36, other reference microbes (listed in Table 1).

Discussion

Detection of a pathogen in nonsymptomatic seedlings is of importance in certification programs for both domestic and international quarantine because a latent population can lead to serious epidemics under favorable conditions. Early detection of diseases is important for assessing the health status of a rice nursery before the transplantation of seedlings to fields.

Recently, development of specific primers and DNA probes from the 16S-23S rDNA spacer region, repeated elements, and siderophore receptor for identification and detection has been reported for *X. oryzae* pv. *oryzae* (16,21). However, it has been revealed that these gene regions are insufficient to discriminate between pathovars of *X. oryzae* in this study because insertion sequence (IS) may undergo periodic extinction in prokaryotic genomes, which are detrimental to their host in the long run, and others are highly conserved in genomes as a level of species (13,18).

A bioinformatics method was used to search specific target sequences for the PCR detection of bacterial pathogens such as a

Fig. 5. Specific detection of *Xanthomonas oryzae* pv. *oryzae* by SYBR Green real-time and conventional polymerase chain reaction (PCR) in infected rice leaves using the primer sets **A**, XO0114F/R and **B**, XO0290F/R, respectively. **A**, Fluorescence intensity corresponding to *rhs* family gene sequences from *X. oryzae* pv. *oryzae* amplified using XO0114F/R. 1, *X. oryzae* pv. *oryzae* KACC10331 genomic DNA; 2–12, inoculated rice leaves; 13–15, healthy rice leaves; 16, no-template control. **B**, PCR amplification of the *rhs* family gene from *X. oryzae* pv. *oryzae* using the XO0290F/R primers. Lane M, size marker (1-kb DNA plus ladder; Gibco BRL); lane 1, *X. oryzae* pv. *oryzae* KACC10331 gDNA; lanes 2–12, inoculated rice leaves; lane 13–15, healthy rice leaves; lane 16, negative control (distilled water).

genome comparing candidate genes with other bacterial DNA genomic sequences by implementing BLAST (22). We found the specific sequence region by sequence alignment search and the *rhs* family genes of *Xanthomonas* and *Pectobacterium* spp. using BLAST and electronic PCR (e-PCR) analysis (11,12). *Rhs* elements were first described in *Escherichia coli* (8); however, their function is as yet unclear. *Rhs* genes are ubiquitous and comprise six structurally distinct lineages within the *Enterobacteriaceae* family. There is considerable intergenomic variation in *rhs* repertoire (4). The *RhsA* core, representative of the *RhsABC* subfamily, diverges by 22% from the *RhsD* core, representative of the *RhsD-E* subfamily (15).

Therefore, in this study, a real-time PCR assay for the detection of *X. oryzae* pv. *oryzae* based on the *rhs* family genes was developed. The results of this study showed that the *rhs* family gene-derived primer pair which has been shown to be specific for *X. oryzae* pv. *oryzae* was amplified (Figs. 2, 4, and 5).

In conclusion, this study indicated that these primer sets are highly specific for *X. oryzae* pv. *oryzae* and that a *rhs* family gene real-time PCR assay can be used as a reliable and useful method for the detection and diagnosis of this pathogen.

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