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Specific Detection of *Xanthomonas oryzae* pv. *oryzicola* in Infected Rice Plant by Use of PCR Assay Targeting a Membrane Fusion Protein Gene

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Successful control of *Xanthomonas oryzae* pv. *oryzicola*, the causal agent of bacterial leaf streak, requires a specific and reliable diagnostic tool. A pathovar-specific PCR assay was developed for the rapid and accurate detection of the plant pathogenic bacterium *Xanthomonas oryzae* pv. *oryzicola* in diseased plant. Based on differences in a membrane fusion protein gene of *Xanthomonas oryzae* pv. *oryzicola* and other microorganisms, which was generated from NCBI (http://www.ncbi.nlm.nih.gov/) and CMR (http://cmr.tigr.org/) BLAST searches, one pair of pathovar-specific primers, XOCMF/XOCMR, was synthesized. Primers XOCMF and XOCMR from a membrane fusion protein gene were used to amplify a 488-bp DNA fragment. The PCR product was only produced from 4 isolates of *Xanthomonas oryzae* pv. *oryzicola* among 37 isolates of other pathovars and species of *Xanthomonas*, *Pectobacterium*, *Pseudomonas*, *Burkholderia*, *Escherichia coli*, and *Fusarium oxysporum* f.sp. *dianthi*. The results suggested that the assay detected the pathogen more rapidly and accurately than standard isolation methods.

Keywords: *Xanthomonas oryzae* pv. *oryzicola*, PCR detection, bacterial leaf streak, rice, membrane fusion protein

Xanthomonas oryzae pv. *oryzicola* is a Gram-negative, rod-shaped bacterium. The pathogen enters through stomates or wounds, multiplies in the mesophyll apoplast, and causes bacterial leaf streak of rice. It is emerging as an important pathogen of rice (*Oryza sativa*) and is a recognized biosecurity threat to the United States. Bacterial leaf streak (BLS) of rice caused by *Xanthomonas oryzae* pv. *oryzicola* has been considered a quarantine disease in Asian countries, including China [16]. Bacterial leaf streak can cause grain weight losses of up to 17%, depending on rice variety and climate condition. The disease is increasing in importance in parts of Asia where modern hybrid rice varieties are grown, as these are particularly susceptible to the pathogen. The disease is widely distributed in tropical and subtropical Asia, including China, Thailand, Malaysia, India, Vietnam, the Philippines, Indonesia, West Africa, South America, and Australia.

Diagnosis of *Xanthomonas oryzae* pv. *oryzicola* infection is based on isolation of the pathogen, followed by biochemical identification, pathogenicity tests, or serological tests [3, 15, 16]. Such tests need one to several weeks before final confirmation is obtained. The sensitivity of the enzyme-linked immunosorbent assay (ELISA) (around 10^6 CFU/ml) is adequate only for detection in symptomatic plants [2]. There are also limitations to the use of these methods in routine seed-health testing of a large volume of seed samples. Cross-reactions with *Xanthomonas oryzae* pv. *oryzae* and other bacteria are observed in some cases.

Research publications related to disease diagnosis over the past ten years indicate rapid movement toward development of DNA based protocols [8, 11, 17]. In contrast, advances in immunodiagnostic methods for bacterial plant pathogens have not been reviewed since 1985 [2]. PCR-based techniques have been reported to be highly efficient for detecting and identifying *Xanthomonas* [12, 14] from plant material. However, only limited information is now available on the molecular detection of plant pathogenic bacteria, and the development of appropriate strategies for their rapid identification and monitoring is needed. The sequence analysis of the 16S rRNA gene and IS elements is widely employed for the identification of bacteria; however, this region is not satisfactorily discriminating between the species or pathovars of *Pseudomonas* [6, 14].

NCBI-BLAST and WU-BLAST searches using the nucleotide sequences of a membrane fusion protein gene from *Xanthomonas oryzae* pv. *oryzicola* BLS256, which are deposited in TIGR's Comprehensive Microbial Resources (http://cmr.tigr.org), found sequence difference at the pathovar level.

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To date, there is no report of such primers for pathovar-specific detection of *Xanthomonas oryzae* pv. *oryzicola*. Therefore, in this study, pathovar-specific primers from a membrane fusion protein gene of *Xanthomonas oryzae* pv. *oryzicola* BLS256 were designed. The primer set showed high specificity for detecting the pathogen in diseased rice.

The comparison of a membrane fusion protein gene (1,268 bp) deposited in the TIGR microbial genome database (TIGR's Comprehensive Microbial Resources: http://cmr.tigr.org/; Locus Name XOCORF_0039) to sequences present in NCBI (http://www.ncbi.nlm.nih.gov/) and CMR (http://cmr.tigr.org/) showed no significant matches with previously determined sequences (BLASTN). BLAST searches with the predicted protein sequence (BLASTX) revealed similarity to colicin V secretion protein [identity=29%, Score=193 bits (490), and Expect=2e-47] from *Xylella fastidiosa*. CMR-BLASTN search showed match to an unnamed protein product [identity=58%, Score=47.6 bits (277), and Expect=0.011] from *Photorhabdus luminescens* TT01. The sequence region to be amplified with the designed primers set (XOCMF/XOCMR) also revealed no significant matches in both BLASTN and BLASTX searches, even in CMR-BLASTN.

The XOCMF and XOCMR primers were tested against *Xanthomonas oryzae* pv. *oryzicola*. As expected, a 488-bp DNA fragment was amplified. To check the specificity of the primers, a large collection of other microorganisms, including *Xanthomonas* species and their pathovars, were tested in PCR assays with primers XOCMF and XOCMR. None of the other *Xanthomonas* strains and reference microorganisms reacted with the primers; only *Xanthomonas oryzae* pv. *oryzicola* showed a single amplified DNA fragment (Fig. 1). The primer pair, XOCMF and XOCMR, amplified a 488-bp DNA fragment from strains of *Xanthomonas oryzae* pv. *oryzicola*, when 50 ng of DNA was used as the template under optimized conditions as described above. The minimum number of cells detected was about 8×10^1 CFU/ml in pure culture suspensions (Fig. 2).

As the primers XOCMF and XOCMR were found to be specific to *Xanthomonas oryzae* pv. *oryzicola*, the sensitivity of the primer pair was tested using seeds of infected rice plant. The primer pair was shown to amplify a 488-bp DNA fragment from the seeds of inoculated rice plants (Fig. 3).

It has been known that many kinds of membrane proteins in the bacterial cell play various and important roles. It was reported that membrane protein was developed for preliminary screening and comparing large numbers of isolates in taxonomic and epidemiological studies [5] and for subtyping *Haemophilus influenza* [9]. In this study, we have investigated membrane proteins to develop more sensitive and specific primer and DNA probes for the detection and identification of *Xanthomonas oryzae* pv. *oryzicola*. The nucleotide sequences of the gene were confirmed for specificity and variety among pathovars within the species through BLASTN and BLASTX searches. In the present study, the primer set (XOCMF/XOCMR) prepared from a membrane fusion protein gene was found to be useful for the specific detection of *Xanthomonas oryzae* pv. *oryzicola* by PCR assay. The nucleotide sequence of a membrane fusion protein gene of *Xanthomonas oryzae* pv. *oryzicola* was also analyzed by BLAST searches and found to be highly variable in different bacteria at the pathovar level (ftp://ftp.tigr.org/pub/data/Microbial_Genomes/x_oryzae_pv_oryzicola/annotation_dbs/). Moreover, the primers XOCMF and XOCMR, which were prepared from a membrane fusion protein gene of *Xanthomonas oryzae* pv. *oryzicola*, were found to be specific for the detection of all strains of *Xanthomonas oryzae* pv. *oryzicola* including different pathovars and species, but not for other phytopathogenic bacteria (Fig. 1), suggesting that the specificity of this primer pair to *Xanthomonas oryzae* pv. *oryzicola* strains may be due to the unique sequence of this pathogen. It is noted that an IS1113 fragment-derived primer pair has previously been reported to be sensitive for the detection of both *Xanthomonas oryzae* pv. *oryzicola* and *Xanthomonas*

Fig. 1. PCR amplification of the partial membrane fusion protein gene from *Xanthomonas oryzae* pv. *oryzicola* using the pathovar-specific XOCMF and XOCMR primer set.
Lane M, Size marker (1 kb plus DNA ladder; Gibco BRL); lanes 1–37 are listed in Table 1.

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

No.	Bacterial and Fungal Isolates	Source	Geographical origin
1	*Xanthomonas oryzae* pv. *oryzicola*	LMG 797	The Philippines
2	*Xanthomonas oryzae* pv. *oryzicola*	BLS 256	The Philippines
3	*Xanthomonas oryzae* pv. *oryzicola*	NCPPB 1585	Malaysia
4	*Xanthomonas oryzae* pv. *oryzicola*	NCPPB 2921	Malaysia
5	*Xanthomonas oryzae* pv. *oryzae*	KACC 10331(K1race)	Republic of Korea
6	*Xanthomonas oryzae* pv. *oryzae*	KACC 10878(K1race)	Republic of Korea
7	*Xanthomonas oryzae* pv. *oryzae*	KACC 10384(K3race)	Republic of Korea
8	*Xanthomonas oryzae* pv. *oryzae*	KACC 10883	The Philippines
9	*Xanthomonas oryzae* pv. *oryzae*	MAFF 311018	Japan
10	*Xanthomonas campestris* pv. *campestris*	ATCC 33913	United Kingdom
11	*Xanthomonas campestris* pv. *carotae*	ATCC 10547	U.S.A.
12	*Xanthomonas campestris* pv. *glycines*	LMG 7403	Zambia
13	*Xanthomonas campestris* pv. *pelargoni*	DSMZ 50857	Germany
14	*Xanthomonas campestris* pv. *malvacearum*	DSMZ 1220	Germany
15	*Xanthomonas axonopodis* pv. *citri*	KACC10443	Republic of Korea
16	*Xanthomonas axonopodis* pv. *dieffenbachiae*	LMG 695	Brazil
17	*Xanthomonas axonopodis* pv. *vasculorum*	LMG 901	Muritius
18	*Xanthomonas axonopodis* pv. *begoniae*	LMG 551	United Kingdom Belgium
19	*Xanthomonas axonopodis* pv. *phaseoli*	LMG 7455	Belgium
20	*Xanthomonas axonopodis* pv. *phyllanthi*	LMG 844	Sudan
21	*Xanthomonas axonopodis* pv. *aurantifolii*	KACC 10161	–
22	*Xanthomonas axonopodis* pv. *vesicatoria*	LMG 905	Tonga
23	*Xanthomonas translucens* pv. *phleipratensis*	LMG 843	U.S.A.
24	*Xanthomonas translucens* pv. *cerealis*	LMG 679	U.S.A.
25	*Xanthomonas translucens* pv. *hordei*	LMG 882	Canada
26	*Xanthomonas arboricola* pv. *poinsettiicola*	LMG 5403	New Zealand
27	*Xanthomonas cassavae*	LMG 673	Malawi
28	*Xanthomonas cucurbitae*	LMG 8662	New Zealand
29	*Xanthomonas theicola*	LMG 8684	Japan
30	*Xanthomonas pisi*	LMG 847	Japan
31	*Pectobacterium carotovorum* subsp. *carotovorum*	LMG 2435	Italy
32	*Pseudomonas syringae* pv. *tomato*	LMG 5093	United Kingdom
33	*Ralstonia solanasearum*	LMG 2299	U.S.A.
34	*Burkholderia gladioli* pv. *gladioli*	LMG 2216	U.S.A.
35	*Burkholderia cenocepacia*	LMG 16656	United Kingdom
36	*Escherichia coli* (O157:H7)	ATCC 35150	–
37	*Fusarium oxysporum* f. sp. *dianthi*	ATCC 11939	–

KACC, Korean Agricultural Culture Collection, Korea (http://kacc.rda.go.kr); NCPPB, National Collection of Plant Pathology, United Kingdom (http://www.ncppb.com); ATCC, American Type Culture Collection, USA; LMG, The Belgian Co-ordinated Collections of Microorganisms (BCCM), Belgium; DSMZ, Deutsch Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany; BLS, International Rice Research Institute, The Philippines (http://www.irri.org).

oryzae pv. *oryzae* [14]. However, in this study, a membrane fusion protein gene's nucleotide sequence-derived primer pair only amplified the DNA of *Xanthomonas oryzae* pv. *oryzicola* strains including *Xanthomonas oryzae* pv. *oryzae* (Fig. 1). Although the successful detection of pathogens using PCR techniques essentially depends on the specificity of the primers, the PCR conditions, including the primers, template, and concentration of Mg^{2+}, thermocyclers, and thermostable polymerase origin have all been shown to affect amplification. Therefore, in this study, all these parameters were optimized to ensure the reproducibility of the amplification. As a result, consistent amplification of a 488-bp fragment from *Xanthomonas oryzae* pv. *oryzicola* was achieved when using different PCR machines and *Taq* polymerase enzymes supplied by various manufacturers, indicating the usefulness of the XOCMF and XOCMR primers for the specific detection of *Xanthomonas oryzae*

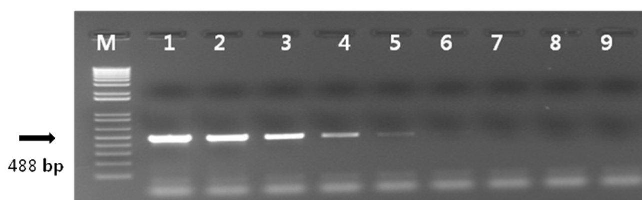

Fig. 2. PCR sensitivity assay for detection of *Xanthomonas oryzae* pv. *oryzicola* using the primer set XOCMF and XOCMR. M. Molecular size marker (1 kb ladder; Invitrogen). Lanes 1–8, dilutions of *Xanthomonas oryzae* pv. *oryzicola* cells ranging from 1.3×10^{8}–1.3 CFU/ml; Lane 9, negative control (distilled water).

Fig. 3. Bio-PCR detection of artificially infected rice seeds.
M, size marker (1 kb DNA plus ladder; Gibco BRL); lane 1, *Xanthomonas oryzae* pv. *oryzicola* BLS256; lanes 2−11, artificially infected rice seeds.

pv. *oryzicola.* Furthermore, the current results clearly demonstrated the sensitivity of the primer pair (XOCMF and XOCMR) for the detection of *Xanthomonas oryzae* pv. *oryzicola* in artificially infected plants. When cell suspensions or tissue extracts are directly used for a PCR, the appearance of unspecific amplification bands is a common problem, which hampers the interpretation of the results. However, no spurious bands were observed in any of the samples analyzed using the XOCMF and XOCMR primers, indicating that this primer set is highly specific to *Xanthomonas oryzae* pv. *oryzicola* and reliable for the detection and diagnosis of this pathogen.

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