Simultaneous Detection of *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in Rice Seed Using a Padlock Probe-Based Assay

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ABSTRACT

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Based on 16S-23S internal transcribed spacer ribosomal DNA sequence data, two padlock probes (PLPs), P-Xoo and P-Xoc, were designed and tested to detect *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, respectively. These PLPs were combined with dot-blot hybridization to detect *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* individually in rice seed. Using this technique, a detection sensitivity of 1 pg of *X. oryzae* pv. *oryzae* genomic DNA was observed. The technique also facilitated the detection of *X. oryzae* pv. *oryzae* in rice seedlots with 2% artificially infested seed. With regards to *X. oryzae* pv. *oryzicola* a

detection threshold of 1 pg genomic DNA was observed and the pathogen was successfully detected in rice seedlots with 0.2% artificially infested seed. The PLP assays detected *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in 39.3% (13 of 33) and 21.3% (10 of 47) of naturally infested commercial rice seedlots, respectively. In contrast, conventional polymerase chain reaction using OSF1/OSR1 and XocF/XocR primers sets detected *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in 9.1% (3 of 33) and 8.5% (4 of 47) of the same rice seedlots, respectively. We also detected both pathogens simultaneously in two seedlots, which successfully proved that PLPs (P-Xoo and P-Xoc) combined with reverse dot-blot hybridization can be used to simultaneously detect multiple pathogens in naturally infested commercial rice seedlots. This approach has the potential to be an important tool for detecting multiple pathogens in seed and thereby preventing the spread of important pathogens.

Bacterial leaf blight (BLB) of rice caused by *Xanthomonas oryzae* pv. *oryzae* (20) and bacterial leaf streak (BLS) of rice caused by *X. oryzae* pv. *oryzicola* (Xoc) (20) are economically important diseases of rice. BLB is one of the most devastating diseases of rice worldwide and has been reported in Asia, Australia, Latin America, and Africa (16,23). It is particularly problematic in irrigated rice production, causing up to 60% yield losses in severe epidemics (21,22). BLS has been reported in tropical Asia, parts of West Africa, and northern Australia (4,15,19,38), and 5 to 30% rice yield losses have been attributed to this disease (28). In 2002, *X. oryzae* pv. *oryzicola* was listed on the U.S. Agricultural Bioterrorism Protection Act as a potential bioterrorism agent, necessitating strict biosecurity measures in addition to standard disease management practices (19). Of the two diseases, BLS is less economically important because its damage potential depends on conditions of high moisture and high rates of nitrogen fertilizer (20,21). Nevertheless, under conducive environmental conditions, BLS can be as destructive as BLB (19).

Infested rice seed is an important source of primary inoculum for *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* (13,25,27); therefore, planting pathogen-free seed is critically important for

managing both diseases. Both BLB and BLS can occur simultaneously on rice plants and the pathogens can contaminate the resulting rice seedlot. Unfortunately, the pathogens are closely related and difficult to distinguish morphologically and genetically (10,16). In fact, *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* show more than 90% similarity by DNA:DNA hybridization (30). Additionally, isolation of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* from rice plants and seed by conventional techniques (e.g., selective agar media) is generally difficult, due to the presence of fast-growing, yellow-pigmented bacterial saprophytes (5).

Polymerase chain reaction (PCR) is an in vitro enzymatic method of amplifying specific DNA sequences (17). It is a highly sensitive and specific technique, which has been used successfully to detect pathogens (11,29,36). Recently, PCR-based assays were developed for the detection of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* (13,27,40). Primer set TXT/TXT4R is widely used and targets an insertion sequence (IS1113) of *X. oryzae* pv. *oryzicola*. Unfortunately, however, *X. oryzae* pv. *oryzicola* DNA yielded false-positive reactions with these primers, because IS1113 is also present in *X. oryzae* pv. *oryzicola* (25). To date, the PCR-based methods have not been widely adopted for commercial seed testing for *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* because of the low tolerance for the pathogens in seed and the presence of seed compounds that inhibit PCR, leading to false-negative results (3,13,27). To overcome this issue, a combined biological and enzymatic amplification technique (BIO-PCR) was developed (25) that substantially improved detection of the pathogen (26). Subsequently, a real-time PCR assay was developed to detect *X. oryzae* pv. *oryzae* and *X. oryzae* pv.

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oryzicola in rice seed (14,41). However, at present, there are few diagnostic techniques available that can detect *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* simultaneously from rice seed. Such a technique would be of great benefit to seed companies and other regulatory agencies because it would reduce the time and seed samples needed to detect both bacteria.

Padlock probes (PLPs) have recently been used to improve detection sensitivity and reduce the cost and time required for plant disease diagnosis (18). PLP-based assays are flexible and easy to adapt to different targets, highly target specific, and suitable for detecting multiple targets (34). PLPs are long oligonucleotides whose ends are complementary to sequences adjacent to the site targeted for PCR amplification. Upon hybridization to the target, the 5' and 3' ends of the PLPs are brought into contact, allowing probe circularization by ligation. Ligation and circularization can only take place when both flanks hybridize to their target sequences with high degrees of fidelity. Noncircularized probes are subsequently removed by exonuclease activity. PLP-based assays are highly applicable for detection of plant pathogens and, recently, this technique was combined with dot-blot hybridization to facilitate detection of *Acidovorax citrulli*, the causal agent of bacterial fruit blotch, in cucurbit seed extracts (33). PLPs combined with universal PCR amplification and microarray detection have also facilitated the simultaneous detection of multiple plant pathogens (31,35).

In this study, we designed two PLPs to target genetic loci unique to *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, and evaluated them for pathogen detection in seed. One approach combined the PLPs with dot-blot hybridization for detecting *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* individually while the second combined the PLPs with reverse dot-blot hybridization for simultaneous detection of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in rice seed.

MATERIALS AND METHODS

Bacterial strains and DNA extraction. In total, 98 bacterial strains, including 33 *X. oryzae* pv. *oryzae* strains, 26 *X. oryzae* pv. *oryzicola* strains, and 39 related species, were obtained (Supplementary Table 1). Genomic DNA was extracted from all strains using a TIANamp Bacteria DNA Kit (TIANamp BIOTECH, Beijing, China) according to the manufacturer's directions.

PLP design. The PLPs were designed as described previously (31) to target unique *X. oryzae* pv. *oryzae* or *X. oryzae* pv. *oryzicola* 16S-23S internal transcribed spacer (ITS) ribosomal DNA (rDNA) sequences. Two 16S-23S ITS sequences from the target pathogens' whole-genome sequences in the National Center for Biotechnology Information (NCBI) GenBank (GenBank accession numbers NC_010717 and NC_017267) were aligned using BioEdit (version 7.0.9.0) (12). The PLPs designed for *X. oryzae* pv. *oryzae* (P-Xoo) and *X. oryzae* pv. *oryzicola* (P-Xoc) were 102 bp long (Table 1) and included regions complementary to the 5' and 3' ends flanking sequences of the *X. oryzae* pv.

oryzae or *X. oryzae* pv. *oryzicola* target regions, respectively. The 5' arm of the PLP was longer than its 3' arm; the 5' arm sequence (T1) was 28 nucleotides (nt) long and the 3' arm sequence (T2) was 14 nt. The universal primer sites and a unique sequence identifier, called Zipcode, were between these segments. The Zipcode sequences were selected from NCBI (GenBank DQ.668361), containing more than five mismatches to known sequences in the public domain (31).

Ligation and exonuclease treatment. *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* genomic DNA digested separately with *Eco*RI, *Hind*III, and *Bam*HI (TaKaRa, Dalian, China) for 30 min, and was used as a template as described below. Cycled ligation was conducted in 10- μ l reactions containing 20 mM Tris-HCl (pH 9.0), 25 mM KCH₃COO, 10 mM Mg(CH₃COO)₂, 10 mM dithiothreitol, 1 mM nicotinamide adenine dinucleotide, 0.1% Triton X-100, 2.4 U of Taq DNA ligase (New England Biolabs, Beijing, China), and 50 pM each PLP (P-Xoo or P-Xoc for *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* DNA, respectively). For simultaneous detection of both pathogens, 10- μ l reactions containing both PLPs were used, and the concentration of each PLP was 50 pM. Reaction mixtures were kept on ice and transferred rapidly into a thermal cycler. After 5 min at 95°C, 20 cycles of 30 s at 95°C and 5 min at 65°C were conducted, followed by 15 min at 95°C to inactivate the reaction. After ligation, 10 μ l of exonuclease mixture (10 mM Tris-HCl [pH 9.0], 4.4 mM MgCl₂, bovine serum albumin at 0.1 mg/ml, 0.5 U of Exonuclease I [TaKaRa], and 0.5 U of Exonuclease III [TaKaRa]) were added to each reaction, and the samples were incubated at 37°C for 15 min, followed by inactivation at 95°C for 3 min.

To determine the specificity of the PLP assay, genomic DNA from 98 bacterial strains was resuspended in a 100- μ l Tris-EDTA (TE) buffer and subjected to cycled ligation followed by PCR amplification with each PLP individually, as described above.

To determine the amplification efficiency of the PLP assay, 10-fold serial dilutions of pure *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* genomic DNA were generated, ranging from 10 ng/ μ l to 100 fg/ μ l, and tested by the PLP assay as described above. For comparison, conventional PCR was conducted using primers OSF1/OSR1 (40) and XoocF/XoocR (40) for *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, respectively. This experiment was repeated five times.

PCR amplification. Amplification of PLPs was carried out in an ABI 2720 thermal cycler (Applied Biosystems, CA) in 25- μ l reactions containing 3 mM MgCl₂, 200 nM dNTP, 300 nM primers P1-F (5'-CCGAGATGTACCGCTATCGT-3') and P2-R (5'-TCATGCTGCTAACGGTCGAG-3'), 1 U of Taq HotStart polymerase (TaKaRa), 0.6 U of uracil DNA glycosylase (New England Biolabs), and 3 μ l of ligation-exo mixture as template. For simultaneous detection of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* DNA, primer P1-F was labeled with digoxigenin (DIG). The reaction mixture was initially incubated at 50°C for 2 min, followed by 10 min of denaturation at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. The reaction was then immediately cooled to 10°C.

TABLE 1. Padlock probes and cZipcode probe used

Probes ^a	Sequence
PLP-Xoo	CACGCGATATAGCTCCGAGGCAACTTGGCTCGACCGTTAGCAGCATGACCGAGATGTACCGCTATCGTGAATCGCCTACAAT TCTGTCTGTACTCCACACAG
PLP-Xoc	GCTCCAAGGCAACTTGGGGTTATATGGTCTCGACCGTTAGCAGCATGACCGAGATGTACCGCTATCGTGC GG CATA CGTT CGT CAAATCAACACGGCATATC
cZip-Xoo	CTTAGCGGATGTTAAGACAG
cZip-Xoc	CGCCGTATGCAAGCAGTTTA
cZip-C1	TAGCCGGACATTAGCCTAGC
cZip-C2	GGCTGAGATCCTAGCACCTA

^a The 5' and 3' ends of the primers that are complementary to the target sequences are indicated by dotted lines. Regions where the two padlock probe-specific primers (P1 and P2) (31) bind for polymerase chain reaction amplification are underlined. The Zipcode sequences, with the unique sequence, specific to the pathogen or target to be detected are in bold. cZipcode probe was the complementary to the Zipcode sequence.

Dot-blot hybridization. Dot-blot hybridization was conducted as described previously (24,33). Briefly, 1 µl of PLP amplicons was spotted onto a specific position on a nylon membrane (Hybond-N⁺; Amersham, Arlington Heights, USA). Ultraviolet cross-linking of DNA to the membrane was carried out for 30 s on a UV hybridizer (UVP HL-2000); then, the membrane was rinsed briefly in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 1% sodium dodecyl sulfate (SDS) for 2 min at room temperature. The membrane was subsequently air dried for approximately 10 min at room temperature. Then, 1 µl of 3 pM cZipcode oligonucleotides was labeled with DIG using a DIG DNA labeling kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instruction. Hybridization was then carried out for 4 h at 42°C. Membranes were washed twice in 2× SSC and 0.1% × SDS for 5 min at room temperature, twice in 0.5% × SSC and 0.1% × SDS for 15 min at 68°C, and once in maleic acid buffer for 2 min at room temperature. To detect hybridization, the membrane was incubated with anti-DIG antibody conjugated with alkaline phosphatase diluted 1:250 in 0.1 M Tris-HCl and 0.15 M NaCl with 1% blocking reagent (Roche Applied Science). After washing the membrane three times in detection buffer (0.1 M Tris-HCl and 0.1 M NaCl, pH 9.5), a substrate consisting of nitroblue tetrazolium-bromochloro-indolyl phosphate (Roche Applied Science) was layered the membrane for 2 to 8 h in the dark. The chromogenic reaction was then stopped by dipping membranes in TE buffer (1 mM EDTA and 1 mM TrisCl, pH 8.0) for approximately 5 min.

Reverse dot-blot hybridization. For simultaneous detection of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, 1 µl of 1 mM cZipcode oligonucleotides was spotted on the nylon membrane (Hybond-N⁺; Amersham) three times. Ultraviolet cross-linking of DNA to the membrane was carried out for 30 s on a UV hybridizer (UVP HL-2000); then, the membrane was rinsed briefly in 2× SSC containing 1% SDS for 2 min at room temperature. The membrane was subsequently air dried for approximately 10 min at room temperature. Then, 5-µl DIG-labeled PLP amplicons were added to 5 ml of hybridization solution (Roche Applied Science) and hybridization was carried out for 4 h at 42°C. The membranes were washed and processed for color detection as described as above.

Detection of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in rice seed. Artificially infested rice seed samples were processed for pathogen extraction as described by Gnanamanickam et al. (9). Briefly, rice seed (5 g) were artificially inoculated by incubation for 1 h in a 200-ml cell suspension containing *X. oryzae* pv. *oryzae* PXO99 or *X. oryzae* pv. *oryzicola* RS105 at approximately 1×10^8 CFU/ml of 0.01 M phosphate-buffered saline (PBS). Control seed samples were treated in a similar way with sterile PBS. Infested seed were then incubated at 4°C for 2 h to permit adsorption of bacteria or buffer onto the seed. Seed was air dried and then mixed with noninfested seed to generate seedlots with 0% (0/1,000 seed), 0.2% (2/1,000 seed) 2% (20/1,000 seed), 5% (50/1,000 seed), and 10% (100/1,000 seed) infestation. Seedlots were incubated with 200 ml of PBS for 4 h on an incubator shaker (Zhicheng, Shanghai, China). Seed samples ($n = 1,000$ seed/lot) were incubated in 50 ml of PBS plus 0.2% Tween 20 for 2 h on a shaker (Zhicheng). The seed extract was decanted and centrifuged at $12,000 \times g$ for 10 min. The supernatant was removed, the pellet was suspended in 1 ml of PBS, and 2 µl of seed extract was used as template for the PLP assay combined with dot-blot hybridization as described above. For comparison, conventional PCR was conducted directly on 2 µl of seed extract of the pellet using primers OSF1/OSR1 (40) and XoocF/XoocR (40) for *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, respectively. This experiment was repeated five times.

Detection of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in naturally infested rice seed samples. To evaluate the ability of the PLP assay to detect *X. oryzae* pv. *oryzae* and *X. oryzae* pv.

oryzicola in naturally infested seed, samples collected from Jiangsu (sample numbers 09Zao-1 to 09Zao-33) and Hainan provinces (sample numbers S1 to S14) were tested as described above. Seedlots that were positive for both pathogens by simplex PLP-based assay were tested again for by duplex PLP combined with reverse dot-blot hybridization. For comparison, samples from the same lots were tested by conventional PCR using primers OSF1/OSR1 (40) and XoocF/XoocR (40). Seed samples ($n = 1,000$ seed) from each lot and the negative control from healthy seed wash were processed as described above, and this experiment was repeated five times. To confirm the results, 100 µl of seed wash from positive seedlots was plated on XOS semi-selective media (5). After incubation for 24 h at 28°C, putative *X. oryzae* colonies were selected for verification by PCR with OSF1/OSR1 and XoocF/XoocR primer sets. To further confirm the identity of the putative *X. oryzae* strains, partial 16S rDNA sequences were generated (TaKaRa) using universal 16S rDNA primers fd2/rp1 (37). These sequence data were used to query the NCBI GenBank database with the BLASTn algorithm (1).

RESULTS

Specificity of PLPs. PLPs in combination with dot-blot hybridization or reverse dot-blot hybridization were applied to differentiate *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*. Hybridization signals generated from the probes P-Xoo and P-Xoc indicated that they were specific to their respective target sequences and facilitated differentiation of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*. None of the 26 *X. oryzae* pv. *oryzicola* strains produced a hybridization signal with P-Xoo. Likewise, none of the 33 *X. oryzae* pv. *oryzae* strains produced a hybridization signal with the P-Xoc assay. As expected, the PLPs did not yield hybridization signals with DNA from 39 related bacterial species.

Sensitivity of PLP-based assays. To determine the sensitivity of the PLP assays, tests were performed using serial 10-fold dilutions (from 10 ng to 100 fg) of pure *X. oryzae* pv. *oryzae* PXO99 and *X. oryzae* pv. *oryzicola* RS105 DNA using the corresponding PLPs in simplex reactions. PCR facilitated the detection of 1 pg of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* genomic DNA (Fig. 1).

Simultaneous detection of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* by PLPs. For simultaneous detection of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, a mixture of the two PLPs was ligated with various template genomic DNAs of *X. oryzae* pv. *oryzae* PXO99 and *X. oryzae* pv. *oryzicola* RS105 (1 pg and 500 pg), treated with exonucleases, and subjected to PCR using DIG-labeled forward primers. The labeled PLP amplicons were analyzed on nylon membranes that allowed the simultaneous detection of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* DNA on a single membrane. The ability of the PLP-based assay to detect two pathogens simultaneously was evaluated. Mixtures of equal concentrations of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* genomic DNAs were tested. The pathogens were correctly and unambiguously identified by both PLPs in 100% of the experiments (Table 2). To evaluate the robustness of the detection assay, we tested the detection threshold for *X. oryzae* pv. *oryzae* PXO99 and *X. oryzae* pv. *oryzicola* RS105 DNA in the presence of high concentrations of nontarget DNA. As little as 1 pg of PXO99 DNA was detected in 100% of the attempts in the presence of 500 pg of RS105 DNA (Fig. 2A and B). Conversely, the detection threshold was 1 pg for RS105 (100% of attempts) in the presence of 500 pg of PXO99 DNA (Fig. 2E and F).

Detection of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in artificially infested rice seed. Conventional PCR detected *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in 40 and 20%, respectively, of rice seedlots with 10% artificially infested seed of each pathogen. Conventional PCR failed to detect either pathogen

in seedlots with lower levels of infestation. In contrast, the P-Xoo-based assay detected *X. oryzae* pv. *oryzae* in 100% of the seedlots with 10, 5, and 2% infestation (Fig. 3A; Table 2). Similarly, the P-Xoc-based assay detected *X. oryzae* pv. *oryzicola* in 100% of rice seedlots with 10, 5, 2, and 0.2% infestation (Fig. 3B; Table 2). Using naturally infested seedlots, the P-Xoo-based assay facilitated the detection of *X. oryzae* pv. *oryzae* in 39.3% (13 of 33) of commercial rice seedlots from Jiangsu (Fig. 4) and the P-Xoc-based assay detected *X. oryzae* pv. *oryzicola* in 21.3% (10 of 47) of commercial rice seedlots from Jiangsu and Hainan (Fig. 5). To confirm the results, seed wash from the seedlots that were positive based on the PLP-based assays were plated on XOS semiselective media. All seed wash yielded corresponding colonies. In contrast, conventional PCR using OSF1/OSR1 primers detected *X. oryzae* pv. *oryzae* in 3 of 33 rice seedlots, and the XocF/XocR primers detected *X. oryzae* pv. *oryzicola* in 4 of 47 rice seedlots.

Simultaneous detection of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in naturally infested rice seed. According to the above results, the PLP-based assay facilitated the detection of both *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* singly from two seedlots, 09Zao-11 and 09Zao-15 (Figs. 4 and 5). To evaluate the ability of the PLP-based assay to simultaneously detect *X.*

oryzae pv. *oryzae* and *X. oryzae* pv. *oryzicola*, samples from 09Zao-11 and 09Zao-15 seedlots were tested using both PLPs with reverse dot-blot hybridization as described above. Using the PLP assays together, *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* were detected simultaneously from both seed samples (Fig. 6).

DISCUSSION

To the best of our knowledge, this is the first report on the application of the PLP-based assay for the simultaneous detection of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in rice seed. Our data provide proof of this concept and indicate that, after further optimization, there is great potential for this technique to be employed for other pathogens in a wide range of seed. Interestingly, the PLP-based assay detected *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in a greater percentage of naturally infected rice seedlots than conventional PCR. The PLP-based assay detected *X. oryzae* pv. *oryzae* in 21.2% of the rice seedlots from Jiangsu, compared with 6.4% by conventional PCR. In a similar manner, the PLP-based assay detected *X. oryzae* pv. *oryzicola* in 39.4% of rice seedlots from Hainan, compared with 18.2% for conventional PCR. All seedlots that were determined to

Fig. 1. Detection of a 10-fold serial dilution of *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* genomic DNA using the corresponding the padlock probes (PLPs). Template DNAs are indicated on the top, while the PLPs are shown on the bottom. NC = negative control, double-distilled H₂O.

TABLE 2. Sensitivity of the padlock probes P-Xoo and P-Xoc

Amount (pg) ^a		Frequency ^b		Xoo infestation ^c	Frequency ^b		Xoc infestation ^d	Frequency ^b	
Xoo	Xoc	P-Xoo	P-Xoc	(%)	PLP assay	Direct PCR	(%)	PLP assay	Direct PCR
500	500	(5/5) 100	(5/5) 100	10	(5/5) 100	(2/5) 100	10	(5/5) 100	(1/5) 100
500	1	(5/5) 100	(5/5) 100	5	(5/5) 100	0	5	(5/5) 100	0
1	500	(5/5) 100	(5/5) 100	2	(5/5) 100	0	2	(5/5) 100	0
1	1	(5/5) 100	(5/5) 100	0.2	0	0	0.2	(5/5) 100	0
1	0	(5/5) 100	0	0.1	0	0	0.1	0	0
0	1	0	(5/5) 100	0	0	0	0	0	0
0	0	0	0	...	...	...	...	...	...

^a Frequency of simultaneous detection of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *X. oryzae* pv. *oryzicola* (*Xoc*) DNA using the padlock probes (PLPs) combined with reverse dot-blot hybridization. Template amount (pg).

^b Detection frequency = the number of positive assays divided by the number of samples tested.

^c Frequency of detection of *X. oryzae* pv. *oryzae* in rice seedlots with different levels of artificial infestation by PLP P-Xoo with dot-blot hybridization and direct polymerase chain reaction (PCR) using primers OSF1/OSR1.

^d Frequency of detection of *X. oryzae* pv. *oryzicola* in rice seedlots with different levels of artificial infestation by PLP P-Xoc with dot-blot hybridization and direct PCR using primers XocF/XocR.

Fig. 2. Detection of genomic DNAs corresponding to complex pathogen DNAs on the nylon membrane using the padlock probes P-Xoo and P-Xoc combined with dot-blot hybridization. Analyzed targets were as follows: **A**, *X. oryzae* pv. *oryzae* PXO99, 1 pg and *X. oryzae* pv. *oryzicola* RS105, 0 pg; **B**, PXO99, 0 pg and RS105, 1 pg; **C**, PXO99, 500 pg and RS105, 500 pg; **D**, PXO99, 1 pg and RS105, 1 pg; **E**, PXO99, 1 pg and RS105, 500 pg; **F**, PXO99, 500 pg and RS105, 1 pg; **G**, double-distilled H₂O; **H**, Layout of targets from each cZipcode oligonucleotides on the membrane.

Fig. 3. Detection of **A**, *Xanthomonas oryzae* pv. *oryzae* and **B**, *X. oryzae* pv. *oryzicola* in rice seedlots with different levels of artificial infestation ranging from 10 to 0% using the corresponding padlock probe (PLP) assay combined with dot-blot hybridization. PLPs are shown on the bottom.

be positive by the PLP-based assay were confirmed by isolation of the respective pathogens on semiselective media and subsequent assays.

Microarray- or macroarray-based multiplex assays that use universal primers to detect variable regions of PLPs have been used to facilitate high-throughput diagnostics (8,35). In fact, because of their high sensitivity and specificity, PLPs have been used to detect single-nucleotide variations in DNA sequences (2,6). When used as a seed health assay, the PLP approach offers numerous advantages over other techniques such as multiplex PCR, which is commonly used for amplifying multiple targets in a single PCR assay (7,32). For example, multiplex PCR is negatively affected by its complexity, low amplification efficiency, and variable efficiency, with different ratios of template concentrations (39). In this study, a mixture of two PLPs was ligated on various template DNAs. After selecting the target complementary

regions, the ligated PLPs were amplified by PCR, which used a pair of universal primers to avoid interference and competition among reactions. The labeled PLP amplicons were analyzed by complementary Zipcode (cZipcode) macroarrays, which enabled the duplex detection of two pathogens on a single nylon. In this study, PLPs resulted in an increase in *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* detection sensitivity over direct PCR. With respect to effectiveness, the PLP-based assay simultaneously also detected *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in rice seed extracts without cross reaction.

A second advantage of the PLP-based approach is the fact that the nylon membrane used is much cheaper than a standard microarray chip. The tag array used in our study contained four cZipcode probes in two replicates with two control probes. Unique Zipcode sequences were selected for each probe. This layout allows for future expansion of the PLP set to target other

Fig. 4. Detection of *Xanthomonas oryzae* pv. *oryzae* in rice seed samples using padlock probe P-Xoo combined with dot-blot hybridization. **A**, Layout of targets from each sample on membrane. **B**, Analyzed samples = 1 to 33, seedlots 09Zao-1 to 09Zao-33; 34 = negative control, the *X. oryzae*-free seed wash; 35 = negative control, double-distilled H₂O; and 36 = positive control, PXO99.

Fig. 5. Detection of *Xanthomonas oryzae* pv. *oryzicola* in rice seed samples using the padlock probe P-Xoc combined with dot-blot hybridization. **A**, Layout of targets from each sample on membrane. **B**, Analyzed samples = 1-33, seedlots 09Zao-1 to 09Zao-33; 34 to 47 = S1 to S14; 48 = negative control, the *X. oryzae*-free seed wash; 49 = negative control, double-distilled H₂O; and 50 = positive control, RS105.

Fig. 6. Detection of *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in rice seed samples using the padlock probes P-Xoc and P-Xoo combined with dot-blot hybridization, simultaneously. **A**, Analyzed sample was 09Zao-11; **B**, analyzed sample was 09Zao-15; **C**, layout of targets from cZipcode oligonucleotides on membrane.

pathogens and thereby enable higher throughput screening. Hence, this approach could be used to test rice seed for a wider array of pathogens, making it a universal rice seed health assay. The only limitations would be the ability to extract adequate quantities of pathogen nucleic acids from seed tissues. Nevertheless, we believe this approach could be applied all seedborne rice pathogens.

A third advantage of the PLP-based assay is its efficiency with regards to the time to assay completion. Including sample processing, the PLP assay required 13 to 19 h to test ≈ 100 rice seed samples ($n = 1,000$ seed/sample). This was substantially less time than the 10 to 14 days required for traditional culturing and subsequent bacterial identification. Although direct PCR could be completed in 3 to 4 h, the PLP-based technique detected a higher percentage of positive seed samples.

Our results proved the concept that PLP-based assays can be used for the simultaneous detection of multiple pathogens in seed. As such, this approach not only could be used for the detection of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* in rice seed but also could serve as a universal assay for all seedborne rice pathogens. As such, this could be an important tool for limiting the dissemination of plant pathogens on rice and other seed.

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