Reliable and Sensitive Detection of *Acidovorax citrulli* in Cucurbit Seed Using a Padlock-Probe-Based Assay

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Abstract

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A method was developed using a padlock probe (PLP) and dot-blot hybridization for detecting *Acidovorax citrulli* in cucurbit seed. The PLP was designed based on the 16S-23S internal transcribed spacer ribosomal DNA sequence from *A. citrulli*. The detection threshold for the PLP assay was 100 fg of genomic DNA, and *A. citrulli* was detected in 100% of artificially infested seedlots with 0.1% infestation or greater. In addition, using the PLP assay, 4 of 8 melon seedlots

collected from Xinjiang province and 15 of 47 watermelon seedlots collected from Ningxia province were positive for *A. citrulli*. In contrast, a conventional polymerase chain reaction (PCR) assay that relied on primers WFB1 and WFB2 facilitated *A. citrulli* detection in 1 of 8 and 5 of 47 seedlots from Xinjiang and Ningxia provinces, respectively. These data indicate that the PLP and dot-blot hybridization technique was more effective than conventional PCR for seed health testing.

Bacterial fruit blotch (BFB), caused by *Acidovorax citrulli* (formerly *A. avenae* subsp. *citrulli*) (19,31), is a devastating seedborne disease of many cucurbitaceous species. Since the first observation in commercial production fields in 1965 (29), BFB has caused millions of dollars of losses in many cucurbit-producing regions of the world (5). Perennially, China produces the greatest number of watermelon and melon crops in the world. However, in recent years, watermelon and melon crops in many provinces have been affected by BFB, including Xinjiang (23), Jilin (10), Neimenggu (9), Gansu (32), Shandong (34), Fujian (6), and Guangdong (17). The disease has caused significant economic losses for both fruit and seed producers.

Infested seedlots are the most important source of primary inoculum for BFB outbreaks (16). Hence, effective BFB management requires that all commercial watermelon and melon seed be tested for freedom from *A. citrulli*. Routinely, cucurbit seed are tested for *A. citrulli* by the seedling grow-out assay or by isolating the pathogen from the seed on semiselective media. However, these assays are generally considered time consuming and inefficient. Recently, polymerase chain reaction (PCR)-based assays were developed for the detection of *A. citrulli* (14,27). Unfortunately, many seeds contain compounds that inhibit PCR and can lead to false-negative results in seed health tests (4,27).

Recently, a diagnostic method was developed based on padlock probes (PLPs) to improve detection sensitivity and reduce the cost and time required for diagnosis (15). PLPs are long oligonucleo-

tides (approximately 100 bp), whose ends are complementary to sequences adjacent to the site targeted for PCR amplification (Supplementary Figure 1). Upon hybridization to the target, the 5' and 3' ends of the PLPs are brought into contact, allowing circularization by ligation. This ligation and resulting circularization can only take place when both flanks hybridize to their target sequences correctly. Noncircularized probes are removed by exonuclease activity. PLPs provide extremely specific target sequence recognition, which is followed by universal amplification and detection by microarray analysis (22). PLPs, combined with universal PCR amplification and microarray detection, have facilitated the simultaneous detection of several important plant pathogens, and all pathogens can be detected using this approach. For example, this approach has been used to detect human-pathogenic viruses (21,28), bacteria (23), and fungi (11) and plant-pathogenic fungi (24). To date, the technique has not been reported for the detection of plant-pathogenic bacteria.

We developed an assay that combines PLP with dot-blot hybridization to detect *A. citrulli* in seed extracts. To detect the bacterium on different samples on a single nylon membrane, the target-specific products were detected by dot-blot hybridization. Briefly, circularized probes were amplified using nonlabeled universal primers, PLP amplicons were spotted onto the nylon membrane, and the cZipCode oligonucleotides were digoxigenin labeled, hybridized to the PLP amplicons, and visualized with a chromogenic assay. In this study, we report the use of the PLP in combination with dot-blot hybridization for reliable and sensitive detection of *A. citrulli* in cucurbit seed.

Materials and Methods

Bacterial strains and DNA extraction. In total, 112 bacteria strains, including 75 strains of *A. citrulli* and 37 related species, were obtained (Table 1). Genomic DNA was extracted from all strains strain using a TIANamp Bacteria DNA Kit (TIANamp BIO-TECH) according to the manufacturer's directions.

PLP design. The PLP designed for *A. citrulli* was 102 bp and contained regions complementary to the *A. citrulli* target region flank at the 5' and 3' ends. Between these segments lie the univer-

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*The e-Xtra logo stands for "electronic extra" and indicates that a supplementary figure is available online and that Figure 1 appears in color online.

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sal primer sites and a unique sequence identifier, called Zipcode. The PLP was designed as described previously (22). The asymmetric 5' arm of the PLP was longer than its 3' arm. The PLP was designed to target unique *A. citrulli* 16S-23S internal transcribed spacer (ITS) ribosomal (r)DNA sequences. Sequences available in GenBank and those obtained from independent sequencing studies were aligned, and sequences unique to *A. citrulli* were selected (Fig. 1). Based on the principles described previously, we designed a PLP, P-Ac (CCGGCGGCACGGTGCAGTTTCCTGCTTTCTCGACCGTTAGCAGCATGACCGAGATGTACCGCTATCGT

CCTTACGCTAGGTCGAGAGTTCAAATTTTGTCA) that was specific to *A. citrulli*. The 5' and 3' ends of the PLP P-Ac, T1 and T2, that are complementary to the target sequences, are indicated by dotted lines. The 5' arm sequence (T1) is 28 nucleotides (nt) long and the 3' arm sequence (T2) is 14 nt long. The regions where the two PLP-specific primers (P₁-F and P₂-R) (22) bind for PCR amplification are underlined. The Zipcode sequence, with the unique sequence specific to the pathogen or target to be detected, is in bold. The unique identifier sequence was selected from an National Center for Biotechnology Information accession

Table 1. Bacterial strains used in this study

No.	Strains	Host	Origin	Reference or source ^a	PLP ^b
<i>Acidovorax citrulli</i>					
1	Njf01	Cantaloupe	Xinjiang, China	Hu Baishi, Nanjing Agricultural University	+
2	Njf02	Cantaloupe	Xinjiang, China	Hu Baishi, Nanjing Agricultural University	+
3	Njl01	Cantaloupe	Xinjiang, China	Hu Baishi, Nanjing Agricultural University	+
4	Xjl04	Cantaloupe	Xinjiang, China	Hu Baishi, Nanjing Agricultural University	+
5	Xjl12	Cantaloupe	Xinjiang, China	Hu Baishi, Nanjing Agricultural University	+
6	Xjl20	Cantaloupe	Xinjiang, China	Hu Baishi, Nanjing Agricultural University	+
7	Btl01	Cantaloupe	Xinjiang, China	Hu Baishi, Nanjing Agricultural University	+
8	Btl02	Cantaloupe	Xinjiang, China	Hu Baishi, Nanjing Agricultural University	+
9	Btl03	Cantaloupe	Xinjiang, China	Hu Baishi, Nanjing Agricultural University	+
10	Cjl01	Cantaloupe	Xinjiang, China	Hu Baishi, Nanjing Agricultural University	+
11	Cjl02	Cantaloupe	Xinjiang, China	Hu Baishi, Nanjing Agricultural University	+
12	Cjl03	Cantaloupe	Xinjiang, China	Hu Baishi, Nanjing Agricultural University	+
13	Fhl01	Cantaloupe	Fuhai, China	Hu Baishi, Nanjing Agricultural University	+
14	Fhl02	Cantaloupe	Fuhai, China	Hu Baishi, Nanjing Agricultural University	+
15	Fhl03	Cantaloupe	Fuhai, China	Hu Baishi, Nanjing Agricultural University	+
16	Anh01	Pumpkin	Anhui, China	Hu Baishi, Nanjing Agricultural University	+
17	Anh02	Pumpkin	Anhui, China	Hu Baishi, Nanjing Agricultural University	+
18	NXF03	Muskmelon	Ningxia, China	Hu Baishi, Nanjing Agricultural University	+
19	NXF04	Muskmelon	Ningxia, China	Hu Baishi, Nanjing Agricultural University	+
20	SD01	Watermelon	Shandong, China	Hu Baishi, Nanjing Agricultural University	+
21	SD02	Watermelon	Shandong, China	Hu Baishi, Nanjing Agricultural University	+
22	NM01	Watermelon	Neimenggu, China	Hu Baishi, Nanjing Agricultural University	+
23	NM02	Watermelon	Neimenggu, China	Neimenggu Agricultural University	+
24	HN01	Watermelon	Hainan, China	Hu Baishi, Nanjing Agricultural University	+
25	HN02	Watermelon	Hainan, China	Hu Baishi, Nanjing Agricultural University	+
26	HB01	Watermelon	Hebei, China	Hu Baishi, Nanjing Agricultural University	+
27	HB02	Watermelon	Hebei, China	Hu Baishi, Nanjing Agricultural University	+
28	HB03	Watermelon	Hebei, China	Hu Baishi, Nanjing Agricultural University	+
29	NX01	Watermelon	Ningxia, China	Hu Baishi, Nanjing Agricultural University	+
30	NX02	Watermelon	Ningxia, China	Hu Baishi, Nanjing Agricultural University	+
31	NX03	Watermelon	Ningxia, China	Hu Baishi, Nanjing Agricultural University	+
32	NX04	Watermelon	Ningxia, China	Hu Baishi, Nanjing Agricultural University	+
33	YN01	Watermelon	Yunnan, China	Hu Baishi, Nanjing Agricultural University	+
34	YN02	Watermelon	Yunnan, China	Hu Baishi, Nanjing Agricultural University	+
35	SY-1	Watermelon	Sanya, China	Henan Agricultural University	+
36	ZZ-2	Watermelon	Zhengzhou, China	Henan Agricultural University	+
37	JL01	Watermelon	Jilin, China	Hu Baishi, Nanjing Agricultural University	+
38	JL02	Watermelon	Jilin, China	Hu Baishi, Nanjing Agricultural University	+
39	Hunan01	Watermelon	Hunan, China	Hu Baishi, Nanjing Agricultural University	+
40	Hunan02	Watermelon	Hunan, China	Hu Baishi, Nanjing Agricultural University	+
41	Hunan03	Watermelon	Hunan, China	Hu Baishi, Nanjing Agricultural University	+
42	JS01	Watermelon	Jiangsu, China	Hu Baishi, Nanjing Agricultural University	+
43	ATCC29625	Watermelon	Georgia, United States	R. Walcott, University of Georgia	+
44	AACAU-9	Cantaloupe	Australia	R. Walcott, University of Georgia	+
45	94-21	Watermelon	Georgia, United States	R. Walcott, University of Georgia	+
46	FC440	Cantaloupe	Xinjiang, China	N. W. Schaad, FDWSRU	+
47	FC464	Cantaloupe	Xinjiang, China	N. W. Schaad, FDWSRU	+
48	00-1	Watermelon	Georgia, United States	R. Walcott, University of Georgia	+
49	94-36	Watermelon	Georgia, United States	R. Walcott, University of Georgia	+
50	94-21	Watermelon	Georgia, United States	R. Walcott, University of Georgia	+
51	92-17	Watermelon	Indiana, United States	R. Walcott, University of Georgia	+
52	94-55	Watermelon	Georgia, United States	R. Walcott, University of Georgia	+
53	94-87	Watermelon	Florida, United States	R. Walcott, University of Georgia	+
54	94-39	Watermelon	Georgia, United States	R. Walcott, University of Georgia	+
55	94-48	Watermelon	Georgia, United States	R. Walcott, University of Georgia	+
56	201-12	Watermelon	South Carolina, United States	R. Walcott, University of Georgia	+
57	94-12	Watermelon	Georgia, United States	R. Walcott, University of Georgia	+
58	203-50	Cantaloupe	Chile	R. Walcott, University of Georgia	+

(continued on next page)

^a FDWSRU = Foreign Disease-Weed Science Research Unit.

^b Padlock probe (PLP) results: + = product of the expected size was produced and – = no product was produced.

(GenBank DQ.668361), containing greater than five mismatches to known sequences in the public domain.

Ligation and exonuclease treatment. *A. citrulli* genomic DNA was digested using *EcoRI*, *HindIII*, and *BamHI* (TaKaRa) for 30 min, and 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 (DTT), 1 mM nicotinamide adenine dinucleotide (NAD), 0.1% Triton X-100, 2.4 U of Taq DNA ligase (New England Biolabs), and 50 pM PLP. 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 performed, 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 albumen 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 112 bacterial strains (Table 1) was extracted using the TIANamp Bacteria DNA Kit (TIANamp BIOTECH) following the manufacturer's instructions, resuspended in a 100- μ l Tris-EDTA

Table 1. (continued from preceding page)

No.	Strains	Host	Origin	Reference or source ^a	PLP ^b
59	206-102	...	China	R. Walcott, University of Georgia	+
60	206-103	...	China	R. Walcott, University of Georgia	+
61	208-27	Watermelon	Indiana, United States	R. Walcott, University of Georgia	+
62	205-14	Watermelon	Indiana, United States	R. Walcott, University of Georgia	+
63	92-301	...	Georgia, United States	R. Walcott, University of Georgia	+
64	92-305	...	Georgia, United States	R. Walcott, University of Georgia	+
65	92-300	...	Georgia, United States	R. Walcott, University of Georgia	+
66	201-18	Watermelon	Australia	R. Walcott, University of Georgia	+
67	98-17	Pumpkin	Georgia, United States	R. Walcott, University of Georgia	+
68	99-5	Cantaloupe	Georgia, United States	R. Walcott, University of Georgia	+
69	200-23	Watermelon	Oklahoma, United States	R. Walcott, University of Georgia	+
70	200-6	Watermelon	Georgia, United States	R. Walcott, University of Georgia	+
71	202-66	Cantaloupe	Israel	R. Walcott, University of Georgia	+
72	203-55	Watermelon	Honduras	R. Walcott, University of Georgia	+
73	207-41	...	China	R. Walcott, University of Georgia	+
74	206-1	...	China	R. Walcott, University of Georgia	+
75	206-2	...	China	R. Walcott, University of Georgia	+
76	<i>A. avenae</i> FC371	Corn	United States	N. W. Schaad, FDWSRU	—
77	<i>A. avenae</i> FC358	Corn	United States	N. W. Schaad, FDWSRU	—
78	<i>A. avenae</i> ATCC19307	Sugarcane	Reunion	China Academy of Inspection and Quarantine	—
79	<i>A. cattleyae</i> NCPB4196	...	...	China Academy of Inspection and Quarantine	—
80	<i>A. cattleyae</i> NCPB4198	...	...	China Academy of Inspection and Quarantine	—
81	<i>A. cattleyae</i> ATCC10200	<i>Cattleya</i> sp.	California, United States	R. Walcott, University of Georgia	—
82	<i>A. facilis</i> A.f-019	...	United States	The United States of America STA	—
83	<i>A. facilis</i> A.f-9503	...	United States	R. Walcott, University of Georgia	—
84	<i>A. konjaci</i> ATCC33996	Konjac	Japan	R. Walcott, University of Georgia	—
85	<i>Xanthomonas oryzae</i> pv. <i>oryzae</i> YN24	Rice	Yunnan, China	Hu Baishi, Nanjing Agricultural University	—
86	<i>X. oryzae</i> pv. <i>oryzae</i> Px099	Rice	Philippines	China Academy of Inspection and Quarantine	—
87	<i>X. oryzae</i> pv. <i>oryzicola</i> JSb3-21	Rice	Jiangsu, China	Hu Baishi, Nanjing Agricultural University	—
88	<i>X. campestris</i> pv. <i>campestris</i> Xcc	Cabbage	Nanjing, China	Hu Baishi, Nanjing Agricultural University	—
89	<i>Erwinia amylovora</i> NCPB1665	Pear	United States	China Academy of Inspection and Quarantine	—
90	<i>E. amylovora</i> IL6	<i>Rubus</i> sp.	Illinois, United States	China Academy of Inspection and Quarantine	—
91	<i>E. pyrifoliae</i> EP28/96	Pear	Korea	China Academy of Inspection and Quarantine	—
92	<i>E. pyrifoliae</i> DSMZ12163	Pear	Korea	China Academy of Inspection and Quarantine	—
93	<i>E. pyrifoliae</i> EjP	Pear	Japan	China Academy of Inspection and Quarantine	—
94	<i>E. tasmaniensis</i> DSMZ17950	Apple	Tasmania, Australia	China Academy of Inspection and Quarantine	—
95	<i>E. billingiae</i> DSMZ17872	...	...	China Academy of Inspection and Quarantine	—
96	<i>E. psidii</i> NCPB3555	...	Brazil	China Academy of Inspection and Quarantine	—
97	<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i> ATCC138	Potato	Arizona, United States	China Academy of Inspection and Quarantine	—
98	<i>Gibbsiella quercinecans</i>	Pear	Jiangsu, China	Hu Baishi, Nanjing Agricultural University	—
99	<i>Dickeya chrysanthemi</i> NCPB2309	Chrysanthemum	Italy	China Academy of Inspection and Quarantine	—
100	<i>Pantoea stewartii</i> subsp. <i>stewartii</i> ATCC8199	Corn	Rugosa, United States	China Academy of Inspection and Quarantine	—
101	<i>P. stewartii</i> subsp. <i>stewartii</i> ATCC8200	Corn	Rugosa, United States	China Academy of Inspection and Quarantine	—
102	<i>P. agglomerans</i> pv. <i>gypsophila</i> DCSS6	Gypsophila	...	South China Agricultural University	—
103	<i>P. stewartii</i> subsp. <i>indologenes</i> DCSS1	...	...	South China Agricultural University	—
104	<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000	Tomato	Guernsey, United Kingdom	China Academy of Inspection and Quarantine	—
105	<i>P. syringae</i> pv. <i>lachrymans</i> Ps18	Cucumber	...	China Academy of Agricultural Sciences	—
106	<i>P. fluorescens</i> Pf	...	Nanjing, China	Hu Baishi, Nanjing Agricultural University	—
107	<i>P. putida</i> Pp	...	Nanjing, China	Hu Baishi, Nanjing Agricultural University	—
108	<i>P. syringae</i> pv. <i>tabaci</i> ATCC11527	...	...	China Academy of Inspection and Quarantine	—
109	<i>P. syringae</i> pv. <i>atropur</i> NCPB1328	...	...	China Academy of Inspection and Quarantine	—
110	<i>Ralstonia solanacearum</i>	...	Nanjing, China	Hu Baishi, Nanjing Agricultural University	—
111	<i>Bacillus cereus</i> Bc	...	Nanjing, China	Hu Baishi, Nanjing Agricultural University	—
112	<i>Escherichia coli</i> Eco	...	...	China Academy of Sciences	—

(TE) buffer, and subjected to cyclized ligation as described above, then PCR amplification. To determine the amplification efficiency of the PLP assay, 10-fold serial dilutions of pure *A. citrulli* genomic DNA were generated at 10 ng/μl to 100 fg/μl and tested by the PLP assay.

PCR amplification. Amplification of the PLP was carried out in an ABI 2720 thermal cycler (Applied Biosystems) in 25-μl reactions containing 3 mM MgCl₂, 200 nM dNTP, 300 nM primers P₁-F (5'-CCGAGATGTACCGCTATCGT-3') and P₂-R (5'-TCATGC TGCTAACGGTCGAG-3'), 1 U of Taq HotStart polymerase (TaKaRa), 0.6 U of uracil DNA glycosylase (New England Biolabs), and 3 μl of ligation-exo mixture as template. 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 and PLP amplicons were analyzed by 2.5% agarose gel electrophoresis at 120 V for 30 min.

Dot-blot hybridization. Dot-blot hybridization was conducted as described by Saiki (18). Briefly, 1 μl of PLP amplicons was spotted onto a specific position on the membrane (Hybond-N⁺; Amersham). 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. cZipcode oligonucleotides (5'-ACTCTCGACCTAGCG TAAGG-3') were digoxigenin-labeled and hybridization was 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-digoxigenin 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-bromo-chloro-indolyl phosphate (NBT-BCIP) (Roche Applied Science) was layered over the membrane. The chromogenic reaction was allowed to proceed for 2 to 8 h in the dark and stopped by dipping membranes in TE buffer (1 mM EDTA and 1 mM TrisCl, pH 8.0) for approximately 5 min.

PLP assay detection of *A. citrulli* in artificially infested seed. Cantaloupe seed (40 g) were artificially inoculated by incubation for 20 min in a 200-ml cell suspension containing *A. citrulli* at approximately 1 × 10⁸ CFU/ml of phosphate-buffered saline (PBS). Seed were then air dried and mixed with noninfested seed to generate seedlots with 0% (0/1,000 seeds), 0.1% (1/1,000 seeds) 10% (10/1,000 seeds), 5% (50/1,000 seeds), and 10% (100/1,000 seeds) infestation. Seedlots were incubated with 200 ml of PBS with Tween (PBST) for 4 h on an incubator shaker (Zhicheng). Seed were processed for pathogen extraction as described by

Walcott et al. (27). Briefly, seed samples (*n* = 1,000 seeds/lot) were incubated in 50 ml of PBST for 4 h on a shaker (Zhicheng). The seed suspension was passed through a triple layer of cheesecloth to remove the seeds and 50 ml of seed wash was pelleted by centrifugation at 6,000 × *g* for 3 min. The pellet was resuspended in 5 ml of PBST and filtered through number 1 filter paper (Whatman). Seed wash (2 μl) 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 wash using primers WFB1 and WFB2 (27). This experiment was repeated three times.

Evaluation of PLP assay for detection of *A. citrulli* in naturally infested seed. To evaluate the ability of the PLP assay to detect *A. citrulli* in naturally infested seed, melon seed samples from markets in Xinjiang province (Xinmiza-1, Xinmi-11, Xinmi-1, Xinmi-32, Xinmiza-7, Xinmi-18, Xinmi-25, and Xinmi-36) and watermelon seed collected from Ningxia province (R001-R047) were tested by PLP assay combined with dot-blot hybridization as described above and conventional PCR using primers WFB1 and WFB2. Seed samples (*n* = 1,000 seeds) from each lot and the negative control from the *A. citrulli*-free seed wash were processed as described above, and this experiment was repeated three times.

To confirm the results, 100 μl of seed wash from positive seedlots was plated on semiselective media TWZ (0.5% peptone,

Fig. 2. Specificity of the *Acidovorax citrulli* padlock probe (PLP) using genomic DNA of *A. citrulli* and *A. avenae*. Ligation of PLP P-ac on genomic DNA of *A. avenae* does not result in a positive signal even in the presence of a very high amount of DNA. Template DNAs are indicated on the top. NC, negative control; M, DL2000 marker (TaKaRa).

Fig. 3. Detection of 10-fold serial dilutions of *Acidovorax citrulli* DNA with the padlock probe assay using agarose gel electrophoresis. NC, negative control; M, DL2000 marker (TaKaRa).

<i>A. citrulli</i> JQ743876	AAA	GCAGGAA	ACTGCA	CCGTGCCGCCG	TGACAAAAATTT	GATTG
<i>A. citrulli</i> GU339092	AAA	GCAGGAA	ACTGCA	CCGTGCCGCCG	TGACAAAAATTT	GATTG
<i>A. citrulli</i> GU339093	AAA	GCAGGAA	ACTGCA	CCGTGCCGCCG	TGACAAAAATTT	GATTG
<i>A. citrulli</i> DQ360418	AAA	GCAGGAA	ACTGCA	CCGTGCCGCCG	TGACAAAAATTT	GATTG
<i>A. a. avenae</i> JQ743887	AAA	GCAGGAA	ACTGCA	CCGTGCCGT	CGGCAACAAAAATTT	GATTG
<i>A. a. avenae</i> EU368726	AAA	GCAGGAA	ACTGCA	CCGTGCCGT	CGGCAACAAAAATTT	GATTG
<i>A. a. avenae</i> JQ743875	AAA	GCAGGAA	ACTGCA	CCGTGCCGT	CGGCAACAAAAATTT	GATTG
<i>A. a. avenae</i> AY080997	AAA	GCAGGAA	ACTGCA	CCGTGCCGT	CGGCAACAAAAATTT	GATTG
<i>A. facilis</i> DQ360425	AAACTGCA	CATTGTTAGT	GAGACCGT	CCGCTAGCA	CAAGAAATTTT	GATTG
<i>A. konjaci</i> GU339095	AAA	GCAGGAA	ACTGCA	CCGTGCCGCCG	TAATTT	GATTG
<i>A. cattleyae</i> AY572032	AAA	GCAGGAA	ACTGCA	CCGTGCCGT	CGGCAACAATAATTT	GATTG
<i>A. cattleyae</i> GU339094	AAA	GCAGGAA	ACTGCA	CCGTGCCGT	CGGCAACAATAATTT	GATTG

Fig. 1. Part of a multiple alignment of 16S-23S internal transcribed spacer sequences of *Acidovorax citrulli* and related bacteria. Symbols: = the target sequence of T1 and -- = the target sequence of T2.

0.025% CaCl₂, 1% Tween-80, berberine at 50 mg/liter, cycloheximide at 50 mg/liter, and 2,3,5-triphenyltetrazolium chloride at 50 mg/liter). After incubation for 24 h at 28°C, putative red *A. citrulli* colonies were selected for verification by PCR with universal 16S rDNA primers fd2/rp1 (30), and amplicons were sequenced (TaKaRa Company). To confirm the identity of putative *A. citrulli* strains from positive seedlots, database searches were performed with the BLASTn algorithm (1).

Results

Testing of the diagnostic PLP. The PLP assay detected all *A. citrulli* strains tested and did not cross-react with nontarget bacteria, including closely related *Acidovorax* spp. (Table 1). In particular, ligation of the PLP P-Ac did not occur with high concentra-

Fig. 4. Detection of 10-fold serial dilutions of *Acidovorax citrulli* DNA with the padlock probe assay using dot-blot hybridization. NC, negative control.

Fig. 5. Detection of *Acidovorax citrulli* in cantaloupe seedlots with different levels of artificial infestation ranging from 10% (spot 1) to 0% (spot 5) using a padlock probe assay combined with dot-blot hybridization. PC, and NC, negative control.

tions of *A. avenae* (100 ng) genomic DNA (Fig. 2). Because *A. avenae* is closely related to *A. citrulli*, these data indicated that the PLP is highly specific.

Sensitivity of PLP assay. The PLP assay detected the target DNA in solutions containing 1 pg of genomic DNA by agarose gel electrophoresis (Fig. 3), and 100 fg of genomic DNA by dot-blot hybridization (Fig. 4). In each of three attempts, the *A. citrulli* genomic DNA was detected at 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg of genomic DNA by agarose gel electrophoresis and dot-blot hybridization. Three attempts using agarose gel electrophoresis failed to detect *A. citrulli* genomic DNA at 100 fg of genomic DNA. In contrast, in all three attempts, the *A. citrulli* genomic DNA was detected at 100 fg of genomic DNA by dot-blot hybridization.

Detection of *A. citrulli* in cucurbit seed. Conventional PCR failed to detect *A. citrulli* in artificially infested cantaloupe seed with varying levels of *A. citrulli* inoculum. In contrast, the PLP assay detected *A. citrulli* in 100% of the seedlots with 10, 5, 1, and 0.1% infestation (Fig. 5).

Using naturally infested seedlots, the PLP assay detected *A. citrulli* in 4 of 8 cantaloupe seedlots from Xinjiang markets and 15 of 47 watermelon seedlots from Ningxia (Fig. 6). To confirm the results, seed wash from the PLP-positive lots was plated on semiselective media TWZ. All of the PLP-positive seed wash yielded *A. citrulli* colonies. In contrast, conventional PCR using WFB1 and WFB2 primers detected *A. citrulli* in 1 of 8 cantaloupe seedlots and 5 of 47 watermelon seedlots.

Discussion

The standard seed health assays for *A. citrulli*, the seedling grow-out assay, and isolation of the pathogen from the seed are time-consuming and inefficient. Immunological assays have also been used but their effectiveness is strongly influenced by the quality of the antibody employed. Additionally, immunological assays are generally less sensitive than PCR-based assays. Microarrays or macroarrays, multiplex detection methods that use universal pri-

Fig. 6. Detection of *Acidovorax citrulli* in **A**, cantaloupe and **C**, watermelon seed samples using a padlock probe assay combined with dot-blot hybridization. **B** and **D**, Layout of targets from each sample on membrane. **B**, Analyzed samples were 1, Xinmiza-1; 2, Xinmi-11; 3, Xinmi-1; 4, Xinmi-32; 5, Xinmiza-7; 6, Xinmi-18; 7, Xinmi-25; and 8, Xinmi-36. **D**, Identifiers R001 to R047 represent watermelon seedlots from Ningxia. PC, positive control and NC, negative control.

mers to detect variable regions of PLPs, facilitate high-throughput diagnostics (8,12,25,26). Because of their high sensitivity and specificity, PLPs have been used to detect single-nucleotide variations in DNA (2,3,7). The homology of the 16S-23S rDNA ITS sequences between *A. citrulli* and *A. avenae* can be as high as 98%. Hence, it is difficult to differentiate these species based on this locus. For instance, one ITS-based PCR primer set designed for *A. citrulli*, SEQID (20), did not perform well at high-stringency conditions, making assays with this primer set more prone to false-positive results. In this study, we designed and tested a PLP for *A. citrulli*. The PLP was designed based on the 16S-23S ITS rDNA sequence from *A. citrulli*. In all, 75 *A. citrulli* strains from six different countries around the world were detected by the PLP assay and the assay did not cross-react with nontarget bacteria, including closely related *Acidovorax* spp.

The assay facilitated detection of the pathogen in naturally infested watermelon and cantaloupe seed. The seedlots that were positive for *A. citrulli* were confirmed by isolation of the pathogen on semiselective media. The PLP assay detected *A. citrulli* in 4 of 8 cantaloupe seedlots from Xinjiang markets and 15 of 47 watermelon seedlots from Ningxia, and the detection of naturally infested seedlots was consistent. In contrast, conventional PCR using WFB1 and WFB2 primers detected *A. citrulli* in 1 of 8 cantaloupe seedlots and 5 of 47 watermelon seedlots. All three replications of the PLP assay on the naturally infested seed lots resulted in positive detections of *A. citrulli*. Plating the seed washes for detection of *A. citrulli* on agar media took considerable time and was subject to false-positive results. Confirmed by isolation of the pathogen on semiselective media, the results of PLP assay were validated, indicating that the PLP assay provides reliable and sensitive diagnosis.

Including sample processing, the PLP assay required approximately 13 to 19 h to test seedlots ($n = 1,000$ seeds/sample). As described by Lunebery et al. (13), dot-blot hybridization was 10 times more sensitive than agarose gel electrophoresis with ethidium bromide staining for detection of *A. citrulli*. Compared with traditional *A. citrulli* seed health assays such as seedling grow-out, the PLP-based assay requires significantly less time. Although dot-blot hybridization increases completion time, it also increases detection sensitivity, which should yield more accurate results.

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