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Novel PCR Primers for Specific Detection of *Xanthomonas citri* subsp. *citri* the Causal Agent of Bacterial Citrus Canker

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ABSTRACT

The new primers were developed for specific detection of *Xanthomonas citri* subsp. *citri* (Hasse) (Xcc) [syn. *X. axonopodis* pv. *citri* (Xac)], the causal agent of Asiatic citrus canker disease. Twenty three strains of Xcc and 34 strains of other xanthomonads including *X. fuscans* subsp. *aurantifolii*, *X. alfalfae* subsp. *citrumelonis*, *X. campestris* pv. *campestris*, *X. campestris* pv. *glycines*, *X. citri* subsp. *malvacearum* and *X. fuscans* subsp. *fuscans* were tested for specificity of new primers by classical PCR. The results showed that these 354 F/R primers specifically amplified all of Xcc strains but not other xanthomonad strains. The 354-bp PCR fragment was sequenced and its nucleotide sequences were compared for similarity with Genbank database. The 354-bp nucleotide sequences were 99.7% similar to gene XAC2443 of Xac strain 306 (Accession AE011881). The sensitivity of these specific primers for detection of viable cells and total DNA of Xcc were 70 CFU/µl and 50 pg/µl, respectively. Therefore, these novel primers can be used as an alternative application for rapid and specific detection of Xcc.
Key words: *Xanthomonas*, bacterial citrus canker, detection, polymerase chain reaction

INTRODUCTION

Bacterial canker of citrus is a serious disease of most citrus species and cultivars in many citrus-producing areas worldwide. Five forms of the disease have been described, cankers A, B, C, D, and E. Canker A or A-strain (Asiatic canker) is the most common and most damaging of the citrus canker strains (Schubert *et al*., 2001). It was originally found in Asia and is by far the most widespread. Recently, information based upon DNA sequences comparison or alignment of 16S-23S internal transcribed spacers (ITS) regions with amplified fragment length polymorphism (AFLP) analysis of the five recognized forms of citrus canker was demonstrated by Schaad *et al*. (2005, 2006). Citrus pathogens were reclassified into three pathovars of *Xanthomonas campestris* (or *X. axonopodis*): pathovars *citri* for strain "A", *aurantifolii* for strains "B/C/D" and *citrumelo* for strain "E", which were revealed as taxon I including all "A" strains; taxon II containing all "B", "C", and "D" strains; and taxon III containing all "E" strains. The taxa I, II and III citrus strains were reinstated with new names, respectively as *Xanthomonas citri* subsp. *citri* (Hasse, 1915),

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Xanthomonas fuscans subsp. *aurantifolii* (Gabriel *et al*., 1989), and *Xanthomonas alfalfae* subsp. *citrumelonis* (Riker *et al*., 1935). A new strain of *X. citri* subsp. *citri*, designated A*, was identified in southwest Asia and has a restricted natural host range to Mexican lime (Verniere *et al*., 1998). Another Aw-strain, which behaves similarly, has recently been discovered in Florida. This strain has a restricted host range that includes Mexican lime and alemow (*Citrus macrophylla*) (Sun *et al*., 2004). The A-strain is the target of international quarantine efforts, in which the development of rapid and reliable procedures for the diagnosis of this pathogen has been a priority.

The polymerase chain reaction (PCR) is a principle for plant disease diagnosis (Henson and French, 1993). However, routine application of PCR for detection of plant pathogens can result in false-positive diagnosis when PCR primers are non-specific to the pathogen. Several sets of primers have been developed for diagnosis of Xcc. Non-specific amplification of Hartung's primers (Hartung *et al.*, 1993) for detection of Xcc were reported by Zaccardelli and Mazzucchi (1997). Miyoshi *et al.* (1998) studied similarity of the intergenic spacer region between 16S-23S rRNA genes among *X. citri* subsp. *citri*, *X. campestris* pv. *glycines*, *X. alfalfae* subsp. *alfalfae* (*X. c.* pv. *alfalfae*), *X. c.* pv. *physalidicola*, *X. c.* pv. *pisi*, *X. c*. pv. *pruni*, *X. c.* pv. *cucurbitae* and *X. c.* pv. *vesicatoria*, and designed primers XCF and XCR based on the data from this study. Primers XCF-XCR were not only for detection of Xcc but also for *X. c.* pv. *glycines*. Kingsley *et al.* (2000) developed fluorogenic PCR assay for specific detection of Xcc A and A*-strain using the forward/reverse primers and probes designed from unique RAPD fragment to target 126 bp amplicon. Mavrodieva *et al.* (2004) designed primers, VM3 and VM4, for real-time PCR by selecting to amplify the *pthA* gene family and run experiments to compare with Kingsley's primers, KF and KR. The results showed that Xcc (A, A* and Aw) and *X. fuscans* subsp. *aurantifolii* (B and C) reacted and gave expected product sizes with VM3-VM4 primers. On the other hand, Kingsley's primers gave prominent band with Xcc A and A*-strain but the reaction with A^w-strain and *X. fuscans* subsp. *aurantifolii* (B and C) were inconsistent and also gave more primer-dimer products when compared with VM3-VM4 primers. The purpose of this study was to design specific PCR primers of Xcc from genomic DNA especially gene XAC2443 (Accession AE011881) in order to apply them for detection of this international quarantine bacterial pathogen of citrus.

MATERIALS AND METHODS

Bacterial strains

To obtain original local strains to be used in this study, canker lesions on lime (*Citrus aurantifolia*), mandarin (*C. reticulata*), sweet orange (*C. sinensis*) and leach lime (*C. hystrix*) were collected from leaves, twigs, and fruit from each major citrus growing area in Thailand. The corky-like raised surface lesions, surrounded by a yellow halo were washed in running water for 1-2 minutes, sprayed with 70% ethyl alcohol, and air-dried. Each lesion was removed from the leaf and cut into 4-5 pieces then soaked in 0.85% NaCl for 20 minutes. A loop of the suspension was streaked onto Fieldhouse and Sasser (FS) agar (Schaad *et al*., 2001) and incubated at 30°C. After 3-4 days, plates were examined for small green-colored starch hydrolyzing colonies typical of Xcc. Promising colonies of Xcc were transferred onto nutrient agar (NA) (Schaad *et al*., 2001) twice and stored either in sterile distilled water at room temperature or on NA slants at 4°C, and in 50% glycerol at -80°C. Several bacterial strains from Japan and the United States of America were included in this experiment (Table 1).

PCR primers

The new primer pair namely 354F-354R

Table 1 Geographical origin, host and year of isolation of strains of *Xanthomonas* species used in this study

Bacterial strain	Geographical origin		Host	Year
X. citri subsp. *citri*				
T1	Kamphaeng Phet	Thailand	*Citrus sinensis*	2003
T3	Chiang Mai	Thailand	*Citrus grandis*	2003
T4	Chiang Mai	Thailand	*Citrus reticulata*	2003
T5	Kamphaeng Phet	Thailand	*Citrus aurantifolia*	2003
T7	Chiang Mai	Thailand	*Citrus reticulata*	2003
T8	Chiang Mai	Thailand	*Citrus reticulata*	2003
T10	Chiang Mai	Thailand	*Citrus grandis*	2003
T13	Kamphaeng Phet	Thailand	*Citrus sinensis*	2003
NT14	Kamphaeng Phet	Thailand	*Citrus reticulata*	2003
NT18	Chiang Mai	Thailand	*Citrus aurantifolia*	2003
NT20	Sukhothai	Thailand	*Citrus aurantifolia*	2003
NT22	Chiang Mai	Thailand	*Citrus grandis*	2003
NT25	Kamphaeng Phet	Thailand	*Citrus reticulata*	2003
OCr1.1	Chiang Rai	Thailand	*Citrus reticulata*	2002
OCr1.2	Chiang Rai	Thailand	*Citrus reticulata*	2002
LCp2.1	Chumphon	Thailand	*Citrus aurantifolia*	2002
LCp2.2	Chumphon	Thailand	*Citrus aurantifolia*	2002
SWRb	Ratchaburi	Thailand	*Citrus sinensis*	2003
Fp1-2	Chiang Rai	Thailand	*Citrus grandis*	2003
XCC-32	Shimizu	Japan	*Citrus natsudaidai*	1998
XCC-131	Yui	Japan	*Citrus unshiu*	1998
1258 (Hartung, Xc-322)		Saudi Arabia	*Citrus* sp.	ND
1270 (Hartung, Xc-328)		Saudi Arabia	*Citrus* sp.	ND
X. fuscans subsp. *aurantifolii*				
1415 (IBSBF 392)		Brazil	*Citrus limon*	1981
1416 (IBSBF 423)		Uruguay	*Citrus limon*	1981
1417 (IBSBF 1583)		Argentina	*Citrus limon*	1990
1418 (IBSBF 380)		Brazil	*Citrus aurantifolia*	1981
1419 (IBSBF 434)		Brazil	*Citrus aurantifolia*	1982
X. fuscans subsp. *aurantifolii*				
1420 (IBSBF 1473)		Brazil	*Citrus aurantifolia*	1999
1421 (IBSBF 1495)		Brazil	*Citrus aurantifolia*	2000
1460		ND	ND	ND
1461		ND	ND	ND
1463		ND	ND	ND
X. alfalfae subsp. *citrumelonis*				
1267 (X-85, J. Miller)		Florida	*Citrus* sp.	1985
1274 (4600, D. Gabriel)		Florida	*Citrus* sp.	ND

Table 1 (continued)

Bacterial strain	Geographical origin		Host	Year
X. citri subsp. *malvacearum*				
1318 (ATCC 14982)		Uganda	*Gossypium hirsutum*	ND
317	Sukhothai	Thailand	*Gossypium hirsutum*	1984
579	ND	Thailand	*Morus sp.*	1986
584	Sukhothai	Thailand	*Gossypium hirsutum*	1986
1034	Nakhon Sawan	Thailand	*Gossypium hirsutum*	1990
1035	Nakhon Sawan	Thailand	*Gossypium hirsutum*	1990
1037	Lop Buri	Thailand	*Gossypium hirsutum*	1990
1051	Loei	Thailand	*Gossypium hirsutum*	1990
1232	Prachin Buri	Thailand	*Gossypium hirsutum*	1993
X. fuscans subsp. *fuscans*				
1316 (NCPPB 381)		Canada	*Phasolus vulgaris*	ND
X. campestris pv. *campestris*				
657	Phetchaburi	Thailand	*Brassica oleracea*	2004
X. campestris pv. *glycines*				
NKR21	Nakhon Ratchasima	Thailand	*Glycine max*	2001
CM 60-1	Nakhon Ratchasima	Thailand	*Glycine max*	2002
No.21-1	Chiang Mai	Thailand	*Glycine max*	2002
RE 07	Khon Kaen	Thailand	*Glycine max*	2002
239	Chachoengsao	Thailand	*Glycine max*	1983
241	Phitsanulok	Thailand	*Glycine max*	1982
281	Phitsanulok	Thailand	*Glycine max*	ND
X. campestris pv. *glycines*				
285	Phitsanulok	Thailand	*Glycine max*	ND
728	Chiang Rai	Thailand	*Glycine max*	1987
1204	Songkhla	Thailand	ND	1992
1324	Songkhla	Thailand	*Vigna radiata*	1994

Abbreviations: IBSBF, Phytobacteria Culture Collection of Instituto Biological, Campinas, Brazil; ATCC, American Type Culture Collection, Manassas, VA; NCPPB, National Collection Plant Pathogenic Bacteria, York, England; ND, not determined.

was designed from *Xanthomonas axonopodis* pv. *citri* strain 306 at section no. 259 from 469 sections of complete genome. This target region resulted from subtractive hybridization (Schaad *et al.*, unpublished). The target at position 4411 to 5228 (partial gene XAC2443) was used for designing new primers for classical PCR by using DNASTAR software (LASERGENE, Version5.1). The sequences of the primers were 354F at position 4675-4693 (5'-GACGGCGCGGCTCAGGATG-3') and 354R at position 5006-5028 (5'-CAGCCCAGCCAACTCAGCACCAG-3'). Other primer pairs also evaluated in this experiment were designed by Kingsley *et al*. (2000), KF (5'-TCCACTGCATCCCACAT CTG-3'), and KR (5'-CAGGTGTACTGCGCTC TTCTTG-3'); Mavrodieva *et al*. (2004), VM3 (5'-GCATTTGATGACGCCATGAC-3'), and VM4 (5'-TCCCTGATGCCTGGAG GATA-3'); and Hartung *et al*. (1993), 2 (5'-CACGGGTGCAAAA AATCT-3'), and 3 (5'-TGGTGTCGTCGCTT GTA T-3') which are respectively referred to as

Kingley's, Mavrodieva's and Hartung's primers in the article.

PCR reaction

PCR was carried out in a 25 µl reaction that consisted of 1x PCR buffer, 3mM $MgCl_2$ for 354 F/R and 2-3 primers and 2mM $MgCl_2$ for KF-KR and VM3-VM4 primers, 0.1 mM dNTPs, 0.6 unit *Taq* DNA polymerase, DNA template 1 µl (50 ng), 0.4 pmole of each primer for 354 F/R, KF-KR and VM3-VM4 primers and 1 pmole for 2-3 primer.

The PCR profiles were designed for each primer as follow: 1.) 94°C for 10 min and 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec and 72°C for 10 min for 354 F/R primers, 2.) 94°C for 10 min and 30 cycles of 94°C for 30 sec, 57°C for 30 sec, 72°C for 60 sec and 72°C for 10 min for KF-KR and VM3-VM4 primers and 3.) 95°C for 10 min and 35 cycles of 95°C for 70 sec, 60°C for 70 sec, 72°C for 60 sec and 72°C for 10 min for 2-3 primers.

Primers specificity tests

Strains of *Xanthomonas* species in Table 1 were used for specificity assay by comparing 354 primers with the Kingsley's, Mavrodieva's and Hartung's primers. Ten microliters of PCR product of each primer was determined by gel electrophoresis on agarose gels in 0.5x TBE buffer at concentration of 1% for 354 bp-PCR primers and 1.5% for Kingsley's, Mavrodieva's and Hartung's primers.

Sensitivity tests

Genomic DNA of Xcc strain T7 was calculated from the absorbance at 260 nm with UV-Visible Spectrophotometer (UV-1601, SHIMADZU®) and adjusted by ten-fold dilution with sterile distilled water from 50 ng to 50 fg for sensitivity tests. Cell suspension of Xcc strain T7 at 0.2 OD of wavelength 600 nm which was about 10^8CFU/ml was also used for sensitivity tests with the ten-fold serial dilutions.

Cloning and sequencing of target DNA fragment

Taq polymerase-amplified PCR products using primer pair 354 F/R were purified and recovered with commercial silica spin column (Promega®). Cloning reactions were according to pCR®8/GW/TOPO® TA Cloning® Kit (Invitrogen®). Briefly, the mixture was incubated at room temperature for 5 min, mixed with *One Shot® Mach*1™-*T1*R *Chemically Competent E. coli* and incubated on ice for 5 min. The cells were transformed for 30 sec at 42°C without shaking and immediately transferred on ice. A 250 µl aliquot of S.O.C. medium were added and incubated on a rotary shaker for 1 hr at 37°C. The transformed cells were centrifuged and suspended in new S.O.C. medium and then 50µl was spread onto LB agar containing 100 µg/ml spectinomycin.

Recombinant clones were screened by PCR amplification with 354 primers, as described above. Sequencing of target DNA product was commercially provided by BSU (Bioservice Unit) using GW1 and GW2 as sequencing primers. The nucleotide sequences were analyzed by the Vector NTI® Advance 9.0 software (Invitrogen®).

Southern blot hybridization: The 354-bp PCR fragment was amplified by using 354 F/R primers and used as the target DNA probe. The method for recovery the DNA fragment from gel was modified from Yue and Orban (2001). The DNA fragment was excised from 0.7% agarose gel in 0.5x TBE with a razor blade. The gel slice was ground with a sterile pestle in a microtube and 300 µl of phenol was added. After vigorously mixing with a vortex, the suspension was centrifuged at 10,000 rpm for 10 min and then 200-300 µl of the supernatant was collected and added to 0.5 volume of 7.5M ammonium acetate and 2.5 volume of absolute ethanol. The supernatant was centrifuged at 10,000 rpm for 15 min and the pellet

was collected and washed with 70% ethyl alcohol. After centrifuging at 10,000 rpm for 10 min, the pellet was dried and suspended in 20-30 µl of sterile distilled water.

The purified target fragment from the previous experiment was labeled with digoxigenin-11-dUTP (Dig-11-dUTP) by using 10xDIG-11-dUTP mixs. The procedure was as follows: the DNA template was diluted to 50 ng and prepared for the 50 µl labeling reaction containing 1µl of DNA template, 5µl of 10x PCR buffer (200mM TrisHCl, 500mM KCl, 20mM $MgCl_2$), 5µl of 10x PCR DIG labeling, 2µl of each 20 pmole/µl primer and 1µl of Taq DNA Polymerase (5 units/µl). The labeling PCR product was separated as described above. The labeled DNA probe was stored at -20°C and denatured by heating in boiling water for 10 min and immediately chilled on ice for 5 min before use.

The agarose gel containing PCR products was depurinated in 0.25% HCl for 30 min and neutralized in 0.4M NaOH for 15 min, and transferred to Highbond N^+ nylon membrane by alkaline 0.4N NaOH. DNAs were fixed under UV transilluminator for 2.5 min to crosslink the DNA to the membrane, and washed as suggested by its manufacturer (Roche®). The membrane was placed into a hybridization bottle containing 3 ml hybridization solution containing 1% blocking solution. After incubating for 1 hr at 65°C the hybridization solution was replaced with a new hybridization solution containing labeled DNA probe and incubated at 65°C for additional 18-24 hr. The membrane was removed and washed on a rotary shaker in solution I (2xSSC, 0.1% SDS) at 65°C for 5 min. The solution was replaced and the membrane was washed for additional 15 min. Finally the membrane was washed twice consecutively with solution II (1xSSC, 0.1%SDS) and solution III (0.5xSSC, 0.1%SDS) for 15 min each at 65°C.

After being washed briefly in washing buffer and 30 min in 1% blocking buffer, the membrane was transferred to anti-digoxigenin alkaline phosphatase conjugated (Roche®) and incubated on a rotary shaker for 45 min at room temperature. The membrane was washed two times with washing buffer for 15 min before being transferred to plastic bag. After adding 500 µl CDP-*Star* solution, the bag was sealed, placed into a Kodak® x-ray cassette and moved to the dark room for the detection step. X-ray film was cut to the proper size and placed over of the membrane and the closed cassette for 30-60 sec. The film was transferred to the developer solution until the band was visible. After washing briefly in water, the film was removed to the fixer solution until the background was clear. Finally the film was washed briefly in water and dried at room temperature before being photographed.

RESULTS

Bacterial strains

X. citri subsp. *citri* strains of Thailand were isolated from different kinds of *Citrus* spp., namely, mandarin, (*C. reticulata*), lime (*C. aurantifolia*), pummelo (*C. grandis*) and sweet orange (*C. sinensis*) from major citrus producing provinces of Thailand (Table 1). Total *X. citri* subsp. *citri* strains in this study were 19 strains from Thailand, 2 strains from Japan, and 2 strains from Saudi Arabia. Other xanthomonads included in this study consisted of 10 strains of *X. fuscans* subsp. *aurantifolii*, 2 strains of *X. alfalfae* subsp. *citrumelonis*, 1 strain of *X. campestris* pv. *campestris*, 11 strains of *X. campestris* pv. *glycines*, 9 strains of *X. citri* subsp. *malvacearum* and 1 strain of *X. fuscans* subsp. *fuscans*.

PCR specificity

The specific 354-bp PCR fragment was amplified with 354 F/R primers from all 23 strains of Xsc (Table 2 and Figure 1A). No fragment of

Table 2 Comparison of specificity test of 354 F/R, VM3-VM4, KF-KR and 2-3 primers by classical PCR. Specific amplification product of each primer pair was determined on agarose gel at concentration of 1% for 354 F/R primers and 1.5% for VM3-VM4, KF-KR and 2-3 primers in 0.5x TBE buffer.

Xanthomonas species	PCR primers			
	354F-354R	VM3-VM4	KF-KR	2-3
X. citri subsp. *citri* (23)[y]	23[z]	23	23	23
X. fuscans subsp. *aurantifolii* (10)	0	5	0	0
X. alfalfae subsp. *citrumelonis* (2)	0	0	0	0
X. citri subsp. *malvacearum* (9)	0	9	0	9
X. fuscans subsp. *fuscans* (1)	0	0	0	0
X. campestris pv. *campestris* (1)	0	0	0	0
X. campestris pv. *glycines* (11)	0	9	0	0

[y] Total number of *Xanthomonas* species in specificity test.

[z] Total number of classical PCR positive results of each *Xanthomonas* species and 0 = negative result.

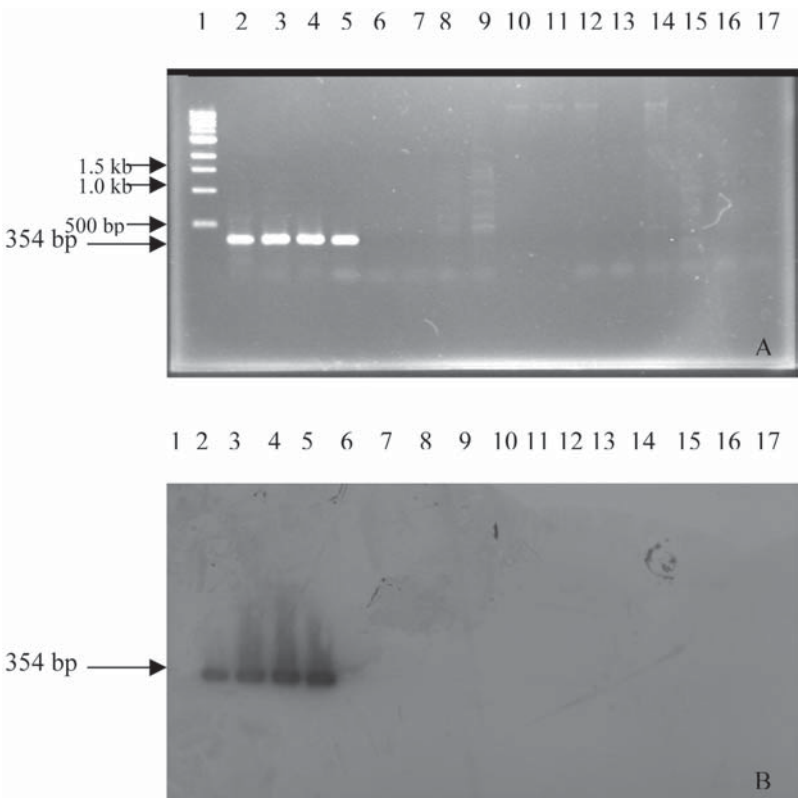

Figure 1 A) PCR amplification products of 354 F/R primers on 1% agarose gel 0.5x TBE buffer. B) Southern blot hybridization with 354 bp probe of *Xanthomonas* species. Lane 1) DNA marker 1 kb (Biolab®); 2-5) *X. citri* subsp. *citri*: T7, J131, 1258, 1270; 6-11) *X. fuscans* subsp. *aurantifolii*: 1415, 1416, 1419, 1420, 1360, 1361; 12-13) *X. alfalfae* subsp. *citrumelonis*: 1267, 1274; 14) *X. campestris* pv. *glycines*: NKR 21; 15) *X. citri* subsp. *malvacearum*: 1318; 16) *X. fuscans* subsp. *fuscans*: 1316; 17) *X. campestris* pv. *campestris*: 657.

expected size was amplified with 354 F/R primers from 34 strains of other xanthomonads including 10 strains of *X. fuscans* subsp. *aurantifolii*, 2 strains of *X. alfalfae* subsp. *citrumelonis*, 9 strains of *X. citri* subsp. *malvacearum*, 1 strain of *X. fuscans* subsp. *fuscans*, 1 strain of *X. campestris* pv. *campestris* and 11 strains of *X. campestris* pv. *glycines* (Table 2).

Other primer pairs, VM3-VM4, KF-KR and 2-3, also amplified expected fragment of PCR product from all strains of Xcc (Table 2). However, VM3-VM4 primers still provided the expected fragment from 5 strains of *X. fuscans* subsp. *aurantifolii* including 1 strain of B-strain and 4 strains of C-strain, 9 strains of *X. citri* subsp. *malvacearum* and 9 strains of *X. campestris* pv. *glycines*. The KF-KR primers were not cross-reacted to other xanthomonads. The 2-3 primers also gave expected fragment from 9 strains of *X. citri* subsp. *malvacearum*.

PCR sensitivity

Sensitivity of 354 F/R primers for detection of viable cells of Xcc and purified total DNA of Xcc strain T7 were 70 cells per µl and 50 pg per µl (Figure 3), respectively by PCR reaction and amplification program were followed as previously.

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Xsc(T7)          1  GACGGCGCGGCTCAGGATGCTGCTAAGGGAGCTGGACGCGCGAAAGGTAATCTGGAAGAC  60
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AE011881.1    4675  GACGGCGCGGCTCAGGATGCTGCTAAGGGAGCTGGACGCGCGAAAGGTAATCTGGAAGAC  4734

Xsc(T7)         61  CAGCTGCGTGTTGCCAACGAGCTACTGCGTGGC*TTGCAAATCCTTGGCATTAGCGACGAA  120
                    |||||||||||||||||||||||||||||||  ||||||||||||||||||||||||||||
AE011881.1    4735  CAGCTGCGTGTTGCCAACGAGCTACTGCGTGGT*TTGCAAATCCTTGGCATTAGCGACGAA  4794

Xsc(T7)         121  GCCGAAGCGTTGGAGCAGGACCTCACCGGGATCTTAAATGCCTTTTCAAAGTCGATTCTG  180
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AE011881.1    4795  GCCGAAGCGTTGGAGCAGGACCTCACCGGGATCTTAAATGCCTTTTCAAAGTCGATTCTG  4854

Xsc(T7)         181  CAAAGTGAAAGAGGGATCGCGACTGCTGAGGAGGCTAGACGCGAGCAGGCTCTCAATACG  240
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AE011881.1    4855  CAAAGTGAAAGAGGGATCGCGACTGCTGAGGAGGCTAGACGCGAGCAGGCTCTCAATACG  4914

Xsc(T7)         241  CTTGTTGCATTTCTAATGAGCTTCGCGAGCCGAAGCGGCGTACGTGATCGACTGAACATC  300
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AE011881.1    4915  CTTGTTGCATTTCTAATGAGCTTCGCGAGCCGAAGCGGCGTACGTGATCGACTGAACATC  4974

Xsc(T7)         301  TTTACCACCAACTATGACAGGCTAATCGAAGCTGGTGCTGAGTTGGCTGGGCTG        354
                    |||||||||||||||||||||||||||||||||||||||||||||||||||||
AE011881.1    4975  TTTACCACCAACTATGACAGGCTAATCGAAGCTGGTGCTGAGTTGGCTGGGCTG        5028
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Figure 2 Comparison of nucleotide sequences of PCR product fragment of 354 F/R primers from *Xanthomonas citri* subsp. *citri* (T7 strain) and *Xanthomonas axonopodis* pv. *citri* strain 306 gene XAC 2443 (Accession AE011881) with BlastN program showed 99.7% similarity
* non-similar nucleotide

Southern blot hybridization

The amplified PCR products from all strains of Xcc (Table 2) were hybridized with 354-bp probe but not with other xanthomonads (Figure 1B).

Sequencing of target PCR product

The 354 bp, expected PCR fragment from Xcc was amplified by 354 F/R primers. The sequences obtained from 354 F/R cloned were blast(N) in Genbank database at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Searching results showed that sequences of 354 bp of expected product were 99.7% similar to sequence of Xac strain 306 gene XAC 2443 (Accession AE011881) (Figure 2).

DISCUSSION

Xanthomonas citri subsp.*citri* (Xcc) is the causal agent of citrus bacterial canker disease, an important pathogen of *Citrus* species, and it is important to international phytosanitary quarantine in many citrus producing countries worldwide (OEPP/EPPO, 2005). Other bacterial citrus pathogens, *X. fuscans* subsp. *aurantifolii* and *X. alfalfae* subsp. *citrumelonis* are closely related to Xcc and *X. alfalfae* subsp. *citrumelo* and have been easily misidentified as Xcc (Schoulties *et al.*, 1987).

Effective control and eradication of citrus canker needs a rapid, specific, and sensitive detection techniques. The polymerase chain

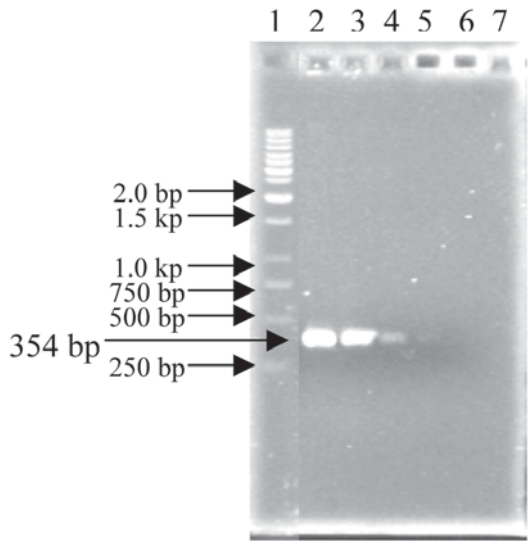

Figure 3 PCR amplification products of 354 F/R primers on 1% agarose gel 0.5x TBE buffer. Lane 1) marker DNA 1 kb (Fermentas?), 2-7) chromosomal DNA of *X. citri* subsp. *citri* at concentration from 50 ng to 50 fg per microliter by ten-fold dilution.

Table 3 Sensitivity of classical PCR for detecting viable cells and purified DNA of *Xanthomonas citri* subsp. *citri* strain T7.

Dilution[a]	Cell/µl	PCR results[c]	DNA concentration[b]	PCR results[c]
0.1 $OD_{600\ nm}$	7.0×10^4	+	50 ng/µl	+
10^{-1}	7.0×10^3	+	5 ng/µl	+
10^{-2}	7.0×10^2	+	500 pg/µl	+
10^{-3}	7.0×10	+	50 pg/µl	+
10^{-4}	7.0	-	5 pg/µl	-
10^{-5}	0	-	500 fg/µl	-
			50 fg/µl	-

[a] Cell suspension of Xcc was adjusted to turbidity 0.1 O.D. of wavelength 600nm which gave 7.0×10^4 cell/µl and ten-fold serially diluted to 10^{-5}. The number of cell per microliter was counted by haemacytometer.

[b] DNA of Xcc was adjusted by ten-fold dilution from 50 ng/µl to 50 fg/µl.

[c] PCR specific amplification with 354 F/R primers were performed following the reaction mix and amplification program in methods. Presence (+) or absence (-) of unique predicted PCR product size after agarose gel electrophoresis.

reaction technique has been used for rapid and reliable detection for many plant pathogens (Henson and French, 1993). Thus, this PCR technique is suitable for routine assay in international quarantine which requires a rapid and sensitive method for routine assay. Plasmid (*pthA* gene family) and chromosomal DNA of Xcc have been used to design specific PCR primers (Hartung *et al.*, 1993, Kingsley *et al.*, 2000, Mavrodieva *et al.*, 2004) for detection of Xcc.

In this experiment, new specific primers, 354 F/R primers, were designed from a fragment in chromosomal DNA of Xcc by substractive hybridization that translated to conserved hypothetical protein (gene XAC2443). The results were that 354 primers showed specific DNA amplification of all strains of Xcc. These Xcc strains were isolated from different hosts and geographical areas in Thailand including strains from Japan and Saudi Arabia which gave the expected 354 bp PCR fragment but not from other xanthomonads (Table 2). This is the first report of using sequences from a conserved hypothetical protein in chromosomal DNA to design specific PCR primers for detection of Xcc. The PCR primers from conserved hypothetical protein region showed more specificity than primers from plasmid DNA (VM3-VM4 and 2-3 primers, Table 2).

The primers designed from chromosomal DNA, KF-KR, had specific amplification with all strains of Xcc in this experiment. The primers also produced prominent band with Xcc A and A*-strain but the reactions with A^w-strain and *X. fuscans* subsp. *aurantifolii* (B and C-strain) were inconsistent and also gave more primer-dimer products (Mavrodieva *et al.*, 2004). At present, PCR product fragment of KF-KR primers still cannot be identified when searching with Genbank database by using BLAST program provided by National Center for Biotechnology Information (NCBI).

The primers designed from plasmid DNA, 2-3 primers and *pthA* gene family, VM3-VM4 primers, amplified not only all strains of Xcc but also other xanthomonad strains. Primers 2-3 cross-reacted with *X. citri* subsp. *malvacearum* and primers VM3-VM4 cross-reacted with *X. fuscans* subsp. *aurantifolii*, *X. citri* subsp. *malvacearum* and *X. campestris* pv. *glycines* (Table 2) because these primers were designed for detection of avirulence or pathogenicity genes which are commonly found in the genus *Xanthomonas* (Gabriel, 1997).

The plasmid DNA has been reported as being easily cured, frequently mutants within the internal sequence and not present in all pathogens (Miyoshi, 1998). The propose of VM3-VM4 primers is to develop universal detection of Xsc and *X. fuscans* subsp. *aurantifolii*. However, results in this experiment showed that the primers did not completely detect all target strains of xanthomonad. They detected one third from *X. fuscans* subsp. *aurantifolii* (B-strain), all 4 strains of *X. fuscans* subsp. *aurantifolii* (C-strain) but did not detect any of *X. fuscans* subsp. *aurantifolii* (D-strain). Nine strains of *X. citri* subsp. *malvacearum* and *X. campestris* pv. *glycines* also reacted with VM3-VM4 primers. The *pthA*, *pthB* and *pthC*, members of *pthA* gene family, belong to a family of avirulence or pathogenicity genes found in the genus *Xanthomonas* (the *avrBs3/pthA* gene family; Leach and White, 1996; Gabriel, 1997) and these may be transferred horizontally on plasmids between Xcc and *X. fuscans* subsp. *aurantifolii* (Brunings and Gabriel, 2003). The results of this experiment also confirmed that the *avrBs3/pthA* gene family is distributed in *X. citri* subsp. *malvacearum* and *X. campestris* pv. *glycines*.

The assay of 354 F/R primers with classical PCR had the ability to detect a lower limit of about 70 CFU/µl of viable cells and the lower limit of detection of 50 pg/µl of purified Xcc total DNA. Sensitivity of other primers to detect viable cells of Xcc and purified Xcc total DNA were 10 CFU/µl and 25 pg/µl for 2-3 and 10 CFU/µl and 1

pg/µl for VM3-VM4 primers. The target PCR product of 354 primers was located in chromosomal DNA of which Xcc carries a single copy per cell, lower than plasmid that Xcc carries multiple copies per cell (Mavrodieva *et al*., 2004). However, the novel 354 F/R primers gave more specific and reliable detection of Xcc than other primers. Real-time PCR techniques, which are based on hybridization of specific probe sequences, are faster and have higher sensitivity and specificity than classical PCR (Schaad *et al*, 2002). Combination of the new specificity primers (354 F/R) with real-time PCR technique will improve the efficacy of Xcc detection to be more specific, sensitive, accurate, reliable, and faster in the future work.

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