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Detection of *Xanthomonas axonopodis* pv. *aurantifolii* and *Xanthomonas axonopodis* pv. *citrumelo* by Triplex PCR

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Citrus bacterial canker is an economically important disease affecting citrus production in many citrus-growing areas and several pathotypes have been recognized within the *Xanthomonas* pathogens causing canker. In view of the containment of the disease, accurate identification of the causal bacterium is important. In this study, triplex PCR method was developed by using the previously reported primers. Two groups of primer combination, such as, one group including primers 2/3, J-pth1/J-pth2 and XACF/XACR, and another group 2/3, J-pth1/J-pth2 and Xac01/Xac02, were suitable for the detection and differentiation of *X. a.* pv. *citri* A^w, *X. a.* pv. *aurantifolii* B and C, and *X. a.* pv. *citrumelo* E strains. Moreover, the primer combination of Xac01 and J-pth2 promised us to use as a specific primer set to detect *X. a.* pv. *citrumelo* E strain. The PCR methods developed in this study could be used for the rapid differentiation of *Xanthomonas* pathotypes of citrus.

Citrus bacterial canker (CBC) caused by *Xanthomonas* spp. has been a serious disease of citrus worldwide (James et al., 2004; Vauterin et al., 1995). The causal agents of CBC have been classified as *X. a.* pv. *citri* A, A* and A^w (syn. *X. citri* subsp. *citri* A, A* and A^w), *X. a.* pv. *aurantifolii* B and C (syn. *X. fuscans* subsp. *aurantifolii* B and C), and *X. a.* pv. *citrumelo* E (syn. *X. alfalfa* subsp. *citrimelonis*) (Parkinson et al., 2009). Due to the economic and legal importance of CBC, PCR methods have been developed for the rapid and reliable identification of the pathogens isolated in culture and from extracts of lesions on leaves and fruits of citrus (Hartung et al., 1993; Cubero et al., 2001). The primer set 2/3 allowed the amplification of a 222 bp DNA fragment of A strains only (Hartung, 1992; Hartung et al., 1993), but failed to detect the A^w strains. Cubero and Graham (2002) reported primer set, J-pth1/J-pth2, based on the *pth* gene, which were reported as the primer that can detect all canker strains. The primer set J-Rxg/J-RXc2 has also been used for identification of

pure culture of A strains. Park et al. (2006) reported *hrpW* specific primer set, XACF/XACR, which producing 561 bp PCR product from *X. a.* pv. *citri* strains. The primer set, Xac01/Xac02 which generating a 581 bp in *X. a.* pv. *citri* A, A* and A^w strains was designed from *rpf* gene cluster of *X. a.* pv. *citri* strain 306 (da Silva et al., 2002, Coletta-Filho et al., 2006).

Multiplex PCR is a variant of PCR in which two or more targets are simultaneously amplified in the same reaction. Since its first description in 1988 (Chamberlain et al., 1988), this method has been successfully applied in detection and diagnosis (Louws et al., 1999), and analyses of polymorphism (Suga et al., 2008). Since there was no trial to apply the method for the differentiation of the citrus pathogens, we tested the previously reported primers to develop multiplex PCR method for the detection and differentiation of the *Xanthomonas* pathogens of citrus.

The CBC related *Xanthomonas* strains were obtained as reference strain from several institutes as described in Table 1. *Xanthomonas* strains were cultured on peptone sucrose agar (1% polypeptone, 1% sucrose and 0.2% L-glutarmate) or LB media depending on the strains. Total genomic DNA was extracted as described by Lee

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Table 1. *Xanthomonas axonopodis* strains used in this study

Type	Strain name	Origin	Source[a]
X. a. pv. *citri* A	CFBP2900	Japan	CIRAD
	M9	USA	DPI
	SL-4510	Korea	PPD
X. a. pv. *Citri* A[*]	IR01	Iran	PPDSI
	JK2-10	Saudi Arabia	CIRAD
X. a. pv. *citri* A[w]	A-2032	USA	DPI
X. a. pv. *aurantifolii* B	CFBP2868, CFBP2903	Argentina	CIRAD
X. a. pv. *aurantifolii* C	CFBP2866	Brazil	CIRAD
X. a. pv. *citrumelo* E	XC05-252, A-1902	USA	DPI

[a]CIRAD: The Agricultural Research Centre for International Development, DPI: Division of Plant Industry, Florida, USA, PPD: Plant Pathology Division, NIAST, Korea, PPDSI: Plant Pests and Diseases Research Institute, Iran.

et al. (2008), and used as template for PCR. Primer sets, 2/3 (Hartung et al., 1993), J-pth1/J-pth2 (Cubero and Graham, 2002), J-Rxg/J-Rxc2 (Cubero and Graham, 2002), XACF/XACR (Park et al., 2006), and Xac01/Xac02 (Coletta-Filho et al., 2006), which have been reported for the detection of *Xanthomonas* pathogens of citrus were preliminarily tested for the optimal PCR conditions. The PCR reaction mixtures contained final concentrations of 50 ng of template DNA, 2.5 mM each dNTP, 2.0 μM of each primer, and 2.0 U of Ex-Taq DNA polymerase with buffer (Invitrogen Corp.). An initial denaturation step at 94ºC for 3 min was followed by 30 cycles of denaturation at 94ºC for 45 s, annealing at 60ºC for 45 s and elongation at 72ºC for 45 s, followed by a final extension incubation of 10 min at 72ºC. Annealing temperature was optimized as 60°C after preliminary test at 58ºC, 60ºC, and 62ºC. Primer set 2/3 detected all of the *X. a.* pv. *citri* A and A* strains. Primer set J-pth1/J-pth2 detected *X. a.* pv. *citri* A, A[*] and A[w] and *X. a.* pv. *aurantifolii* B and C

with variation in B strains. Primer sets, XACF/XACR, J-Rxg/J-Rxc2 and Xac01/Xac02 detected all of the *X. a.* pv. *citri* A, A[*] and A[w]. No amplification product was obtained with *X. a.* pv. *citrumelo* E strain by any of the primers tested (Fig. 1). The primer set, 2/3, which were previously reported to detect A strains from A[w] strains differentiated A[w] strains from both A and A[*] strains. The primer set based on the *pth* gene were originally reported to detect all canker strains with slight variation for the detection of *X. a.* pv. *aurantifolii* B strains (Cubero and Graham, 2002). The variation for the detection of B strain was also shown in this experiment. The other primer sets, J-Rxg/J-Rxc2, XACF/XACR, and Xac01/Xac02, were suitable for the detection of A strains and its variants, A[*] and A[w] strains.

To confirm the pathotype of CBC strains, it is necessary to isolate the bacterium from lesions and to perform a pathogenicity test on several citrus plants. And additional tests should be experimented if any deviations from regular host range were found. To develop reliable method for the differentiation of the *Xanthomonas* pathotypes by multiplex PCR, we tested two combinations of primer sets, namely, one group; primers 2/3, J-pth1/J-pth2 and XACF/XACR (Fig. 2A) and another group; primers 2/3, J-pth1/J-pth2, and Xac01/Xac02 (Fig. 2B). Triplex PCR was performed as described above except 25 ng of each template and 0.1 μM of each primer, and aliquots of 5 μl from amplification reactions were subjected to electrophoresis through 3.0% (w/v) agarose gels. Two groups produced similar amplification bands as expected and differentiated *X. a.* pv. *citri* A from *X. a.* pv. *aurantifolii* B, C and *X. a.* pv. *citrumelo* E by different amplications between primers J-pth1/J-pth2 and XACF/XAR, J-pth1/J-pth2 and Xac01/Xac02 in the first and second group, respectively. *X. a.* pv. *citri* A[w] was also differentiated by the difference of detection between primers 2/3 and XACF/XACR, and 2/3 and Xac01/Xac02, in the first and second group, respectively. The results indicated that *X. a.* pv. *citri* A[w], *X. a.* pv.
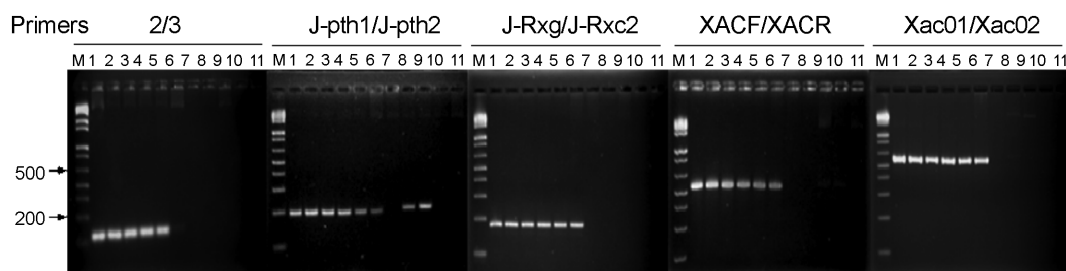

Fig. 1. Specific amplification of *Xanthomonas* strains by PCR. PCR reaction was performed at 60ºC of annealing temperature. Lane M: 1-kb plus DNA ladder; lanes 1-3: CFBP2900, M9, and SL-4510 strain of *X. a.* pv. *citri* A, lanes 4-5: IR01 and JK2-10 of *X. a.* pv. *citri* A[*], lane 6: A-2032 of *X. a.* pv. *citri* A[w], lanes 7-8: CFBP2868 and CFBP2903 of *X. a.* pv. *aurantifolii* B; lane 9: CFBP2866 of *X. a.* pv. *aurantifolii* C; lanes 10-11: XC05-252 and A-1902 of *X. a.* pv. *citrumelo* E. The arrow indicates the size of 200 and 500 bp.
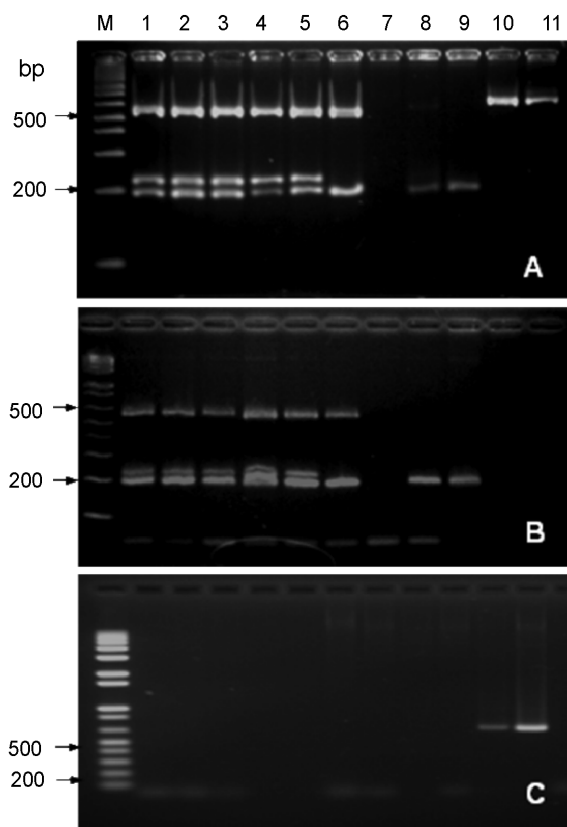
Fig. 2. Triplex PCR performed for the differentiation of *Xanthomonas* strains. The combinations of primers, (**A**) 2/3, J-pth1/J-pth2 and Xac01/Xac02, (**B**) 2/3, J-pth1/J-pth2 and XACF/ XACR, and (**C**) primer set Xac01/J-pth2 were used for the PCR. The same strains as described in Fig. 1 were used for each lane .

aurantifolii B and C, and *X. a.* pv. *citrumelo* E could be differentiated by one PCR reaction.

Non-specific PCR products were produced from the template of *X. a.* pv. *citrumelo* E strain in the first combination of primers (Fig. 2A). To find out the specific primers producing the bands from E strain, we used alternate combinations of forward and reverse primers from the first combination. The resulting bands were produced from the primer set, Xac01/J-pth2 when the gDNA of E strain was used as template (Fig. 2C). This result strongly indicated that the primer set, Xac01/J-pth2, could be used for the detection of E strains from the other *Xanthomonas* strains of citrus. Freshly grown bacterial cells were washed 2 times with 5 M NaCl, and a concentration of 10^7 CFU/ml in sterilized distilled water was used as PCR template. The result in this test indicated that the triplex PCR could be applied directly to differentiate the pathotypes right after bacterial isolation (data not shown). However, the detection ability was seriously decreased when the concentration was below 10^5 CFU/ml.

PCR-based diagnostic test is suitable for monitoring pathotypes in a very short time compared to laborious, sophisticated and expensive protocols. The triplex PCR method developed in this study could be rapid and reliable approach for differentiation of *Xanthomonas* pathotypes of citrus in quarantine situation. Furthermore, the protocol presented in this study could be used for routine diagnosis to aid in the study of CBC epidemiology, and to improve the management of canker disease in the citrus fields.

Acknowledgement

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