Simultaneous Detection of Four Citrus Graft-transmissible Pathogens in the People's Republic of China

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ABSTRACT. Multiple infections of more than one graft-transmissible pathogen often occurs in field citrus. In this paper, a simple and sensitive one-step multiplex (RT)-PCR protocol plus an internal control has been developed to detect four citrus graft-transmissible pathogens: *Candidatus* Liberibacter asiaticus (*Ca.* L. asiaticus), *Citrus tristeza virus* (CTV), Citrus tatter leaf virus (CTLV), and *Citrus exocortis viroid* (CEVd). The sizes of specific bands amplified from these four pathogens are 1160 bp for *Ca.* L. asiaticus, 511 bp for CTV, 371 bp for CEVd and 309 bp for CTLV. This study was addressed to rapid detection of multiple pathogens in field trees, and further to facilitate evaluating the exclusion effect of multiple pathogens for virus-free propagation in China. Our data showed that most field trees were infected with CTV, whereas co-infections occurred usually with no more than two pathogens, while a few plants harbored up to three pathogens. Of samples collected from coastal areas, co-infections with CTV and HLB occurred very often, but seldom with CTV and CTLV.

Five citrus graft-transmissible pathogens have been experimentally confirmed in China, including *Candidatus* Liberibacter asiaticus (*Ca.* L. asiaticus) associated with Huanglongbing (HLB), *Citrus tristeza virus* (CTV), *Citrus tatter-leaf virus* (CTLV), *Citrus exocortis viroid* (CEVd) and *Satsuma dwarf virus* (SDV). These pathogens induce tristeza, tatter-leaf, exocortis, and satsuma dwarf diseases, respectively. However, HLB is still the main threat to the coastal citrus production areas. Severe tristeza stem-pitting is another threat to sweet pummelos and oranges. Though CTLV and CEVd were less widespread than CTV and *Ca.* L. asiaticus, the two pathogens respectively cause tatter-leaf and exocortis in citrus cultivars grafted on trifoliate orange (*Poncirus trifoliata*) or its hybrids, which are widely used in China as rootstocks. Multiple infections of more than one graft-transmissible pathogen often occurs in field trees.

For the detection of citrus pathogens, biological indexing is a classical diagnostic method (5). However, this method is time-consuming, and requires specialized facilities, such as a greenhouse or screenhouse. The polymerase chain reaction (PCR) is a powerful method and is the most commonly employed technique for its sensitivity and specificity. Simplex PCR detects one target per reaction and requires different PCRs to detect multiple targets. Multiplex PCR (mPCR) is a useful technique since different pathogens can be detected simultaneously (4)**.** In this paper, since SDV is seldom distributed in field trees due to the use of virus-free materials, detection of the other four pathogens simultaneously and efficiently is of great importance. Thus, a simple, sensitive and one-step multiplex (RT)-PCR protocol for these pathogens plus an internal control has been developed.

Leaf or bark (10–15 mg) from each of 28 field trees was put into a 1.5 ml Eppendorf tube immersed in liquid nitrogen and total nucleic acids were extracted as described previously (7, 8). The primer pairs according published methods (1, 2, 3, 6) were used. Because the ubiquitin gene is always expressed at a higher stability level than most of housekeeping genes, it was

selected for an internal control in this study (1). Various parameters were considered to optimize the mPCR reactions, especially for primer concentration and annealing temperature. Amplifications were carried out in a 10 µl reaction mixture with the SuperScript™ one-step RT-PCR system with Platinum Taq DNA polymerase kit (Invitrogen). The reaction was performed with the mixture of all pathogens' primer pairs at a final concentration of 0.16 µM for CTLV, 0.5 µM for CEVd, 0.4 µM for HLB and 0.13 µM for CTV. Primers to the ubiquitin gene were used as an internal control (UBQ-F, -R, each at 0.24 µM). The optimum one-step protocol consisted of 1 µl of total nucleic acids added to 9 µl of RT-PCR mixture containing: 5 µl 2x buffer; 0.3 µl 50mM MgSO4; 0.3 µl 10mM dNTPs; 0.6 µl 100 mM DTT; 1.43 µl of primers; 0.4 µl of RT-Taq; 0.97 µL of sterile water. Optimal one-step multiplex RT-PCR parameters were an initial incubation at 55°C for 30 min for cDNA synthesis, followed by denaturation of the RT enzyme for 5 min at 94°C and then, 35 cycles of denaturation at 94°C for 30 s, primer annealing and extension at 68°C for 80 s followed by final extension at 68°C for 7 min. The amplified PCR products were analyzed on 1.5% agarose gels stained with ethidium bromide. In order to confirm the specificity of the multiplex PCR protocol, PCR products were purified and subjected to sequence analysis. As expected for each pathogen, the DNA sequences were obtained (data not shown).

Fig.1 shows the amplification results for single infection extracts, individually or combined, in the multiplex (RT)-PCR system. No false-positives were observed in any of the samples tested. The expected product for the internal control was detected in all samples analyzed. The four expected products were detected when the mixture of four single infection extracts was analyzed by multiplex (RT)-PCR (Fig. 1, lane 1).The sizes of specific bands amplified from the four pathogens are 1160 bp for HLB, 511 bp for CTV, 371 bp for CEVd and 309 bp for CTLV.

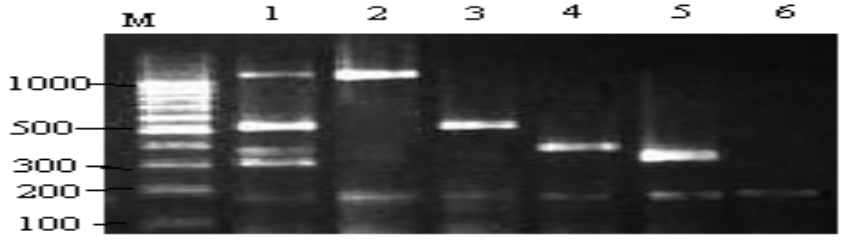

Fig. 1. One-step multiplex (RT)-PCR analysis of single infection extraction extracts. *Lane1* mix of total nucleic acids tested in lanes2-5; *Lane2* HLB*; Lane3* CTV; *Lane4* CEV; *Lane5* CTLV; *Lane6* healthy control. M.100bp molecular marker.

Table1 shows the results obtained from 28 representative citrus trees comprising of sweet orange, lemon, mandarin and mandarin hybrid. The results obtained by one-step multiplex (RT)-PCR corresponded exactly to those single (RT)-PCR (data not shown). The citrus samples of different varieties investigated in the study were local cultivars. Most field trees were infected with CTV in the field. The distribution of CEVd and CTLV in China is sporadic. Of the samples collected from the coastal areas, co-infections occurred usually with no more than two pathogens, while a few plants

harbored up to three pathogens. Moreover, co-infections with CTV and HLB occurred often, but seldom with CTV and CTLV. In addition shoot-tip grafting budlings were quickly assayed by this method, most were confirmed virus-free (data not shown).

The rapid and specific one-step multiplex (RT)-PCR assay is a rapid method for detection of these four important pathogens of citrus in field and for evaluating the exclusion effect of multiple pathogens for virus-free propagation.

TABLE 1
ONE-STEP MULTIPLEX (RT)-PCR ANALYSIS FOR FOUR PATHOGENS OF 28 FIELD SAMPLES FROM SWEET ORANGES, MANDARINS, MANDARINS HYBRIDS AND LEMONS IN CHINA

No.	Cultivar	Multiplex (RT)-PCR				
		Origin of samples	CTV	CEVd	CTLV	HLB
1	Shatang mandarin	Yunnan	+	-	-	-
2	Shatang mandarin	Yunnan	+	-	-	+
3	Eureka lemon	Yunnan	-	-	-	-
4	Eureka lemon	Yunnan	+	-	-	+
5	Xingguo sweet orange	Jiangxi	+	+	-	-
6	Newhall navel orange	Jiangxi	+	+	-	-
7	Hamlin sweet orange	Jiangxi	+	-	-	-
8	Newhall navel orange	Jiangxi	-	-	-	+
9	Miyagawa wase unshiu	Hunan	+	-	-	-
10	Ehime kashi No.22	Hunan	+	-	-	-
11	Ehime kashi No.28	Hunan	+	-	-	-
12	Nankan unshiu No.20	Hunan	+	-	-	-
13	Xiangshanhong tangor	Zhejiang	+	-	-	-
14	Dafen unshiu	Zhejiang	-	-	-	-
15	Amakusa Tangor	Chongqing	+	-	-	-
16	Fuzaocheng sweet orange	Chongqing	+	+	+	-
17	Yuhongcheng sweet orange	Chongqing	+	-	-	-
18	fengyuan No.72-1 navel orange	Chongqing	+	+	-	-
19	Powell navel orange	Chongqing	+	-	+	-
20	Summer gold navel orange	Chongqing	+	-	-	-
21	xinhuicheng sweet orange	Guangdong	+	+	-	+
22	Anliucheng sweet orange	Guangdong	+	-	-	+
23	Shatang mandarin	Guangdong	+	-	+	+
24	Shatang mandarin	Guangdong	+	-	-	-
25	Anliucheng sweet orange	Guangxi	+	-	-	-
26	Seike navel orange	Guangxi	+	-	-	+
27	Seike navel orange	Guangxi	+	-	-	-
28	Anliucheng sweet orange	Guangxi	+	-	-	+
	Number of positive(%)		25(89.3)	5(17.9)	3(10.7)	8(28.6)

ACKNOWLEDGEMENTS

This study was supported by the Program for Changjiang Scholars and Innovative Research Team in University (PCSIRT, IRT0976), the National Natural Science Foundation of China (30600419) and the Fundamental Research Funds for the Central Universities (XDJK2009C133).

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