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A rapid one-step multiplex RT-PCR assay for the simultaneous detection of five citrus viroids in China

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Abstract Citrus plants are natural hosts of five viroid species and large numbers of sequence variants. In this paper a simple and sensitive one step multiplex RT-PCR protocol with an internal control was utilised to simultaneously detect and differentiate five citrus viroids: *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid-III* (CVd-III) and *Citrus viroid-IV* (CVd-IV). In addition, a micro and rapid total nucleic acid extraction method was developed and the protocol applied to evaluate the occurrence and distribution of citrus viroids in China.

Keywords Citrus viroids · Multiplex · One-step RT-PCR · Nucleic acid extraction method

Viroids, the smallest known agents of infectious disease, are small (246–401 nts), covalently closed single-stranded RNAs that completely depend on host enzymes for replication (Diener 2001; Flores et al. 2005). Viroids have been classified into two families, *Pospiviroidae*, composed of species with a Central Conserved Region (CCR) and without hammerhead ribozymes, and *Avsunviroidae*, composed of three members lacking CCR but able to self-cleave through hammerhead ribozymes.

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Citrus trees are natural hosts of at least five viroid species of the family *Pospiviroidae*: *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid-III* (CVd-III) and *Citrus viroid-IV* (CVd-IV) (Duran-Vila et al. 1988, 2000). In general, CEVd and specific variants of HSVd induce exocortis and cachexia diseases, respectively on sensitive citrus hosts (Semancik and Weathers 1972; Semancik et al. 1988). In addition, some CBLVd, HSVd, and CVd-III variants can induce dwarfing in citrus cultivars grafted on trifoliate orange (*Poncirus trifoliata*) rootstock (Ashulin et al. 1991; Semancik et al. 1997; Hutton et al. 2000). CVd-IV, which appears to be less widespread than the other four viroids, can cause severe bark cracking on susceptible species (Puchta et al. 1991). More recently, other two new viroid species have been characterised: *Citrus viroid-OS* (CVd-OS), reported in Japan and *Citrus viroid V* (CVd-V), isolated from *Atalantia citroides* in Spain (Ito et al. 2001; Serra et al. 2008).

Indicator indexing and sequential polyacrylamide gel electrophoresis (sPAGE) analysis are classical diagnostic methods for citrus viroids (Roistacher et al. 1977; Duran-Vila et al. 1993). However, these methods are time-consuming, and require specialised facilities, such as greenhouses or screenhouses. A multiprobe assay based upon molecular hybridisation for the simultaneous detection of multiple citrus viroid infection has been developed, but reverse transcription (RT)-PCR and multiplex RT-PCR are the most popular and reliable assays for their great

sensitivity and specificity (Cohen et al. 2006; Yang et al. 1992; Levy et al. 1992; Tessitori et al. 1996; Ito et al. 2002).

A multiplex RT-PCR for the simultaneous detection of citrus viroids and *Apple stem grooving virus* (ASGV) has been established by Ito et al. (2002). In the present study we have developed a one-step multiplex RT-PCR for the detection of CEVd, CBLVd, HSVd, CVd-III and CVd-IV, plus an internal control, using redesigned or existing primer sets, and using a mini-prep and rapid total nucleic acid extraction procedure. Citrus samples were collected from eight provinces in China and maintained in a screenhouse of the National Citrus Virus Exclusion Center (NCVEC). Some positive control materials (CBLVd, HSVd and CVd-III isolates) were kindly supplied by Prof. P. Barkley from Elizabeth Macarthur Agricultural Institute (EMAI), NSW Department of Primary Industries, Australia. CVd-IV isolates were kindly supplied by Dr. N.Önelge from Subtropical Fruits Research and Experimental Centre, University of Çukurova, Turkey.

Total nucleic acids were extracted as described previously (Zhou et al. 2001). Leaf, bark, fruit skin or root (5–10 mg) was put into 1.5 ml Eppendorf tube immersed in liquid nitrogen, then ground with a sterile plastic pestle (BioRad) and homogenised with 60 μl TES buffer (100 mM Tris–HCl, 2 mM EDTA and 2% (*w*/*v*) SDS) and 60 μl of a mixture composed by water-saturated phenol/chloroform/isoamyl alcohol (25:24:1 *v*/*v*/*v*). The homogenised samples were incubated in a water bath at 70°C for 5–10 min, followed by centrifugation at 12,000×*g* for 5 min. During this time, a single hole was made with a heated 25 gauge needle in the bottom of a 0.5 ml Eppendorf tube and a small quantity of glass beads (425–600 μM, Sigma) maintained in TNE buffer (10 mM Tris–HCl, 100 mM NaCl, 1 mM EDTA) was added for covering the hole. This mini-column was then filled with a slurry of Sephadex G-50–80 (Amersham Biosciences) preequilibrated with the above TNE buffer, placed inside a 2.2 ml centrifuge tube and spun at 5,000×*g* for 3 min to pack the matrix. The packed mini-column was then placed into a sterile 1.5 ml Eppendorf tube, and 40 μl of aqueous phase of the extract was transferred into the matrix and the pellet was discarded. Finally, the mini-column was then centrifuged at 5,000×*g* for 4 min to retrieve the eluate (about 20 to 80 μl). Total nucleic acid extracts were used directly for RT-PCR or stored at −20°C for periods of up to 1 year. Extracted RNA purity was assessed by spectrophotometer (BioRad SmartSpec™ Plus) and the OD260/OD280 ratio of all extracts ranged from 1.6 to 1.8. The extraction protocol has several advantages: (1) it does not require special commercial kits and it is rapid, low cost and easy to handle; (2) all manipulation in Eppendorf tubes minimises contamination risks; (3) it needs only a 5–10 mg sample.

The primer pairs used are listed in Table 1. Amplifications were carried out in a 10 μl reaction mixture with the SuperScript™ one-step RT-PCR system with Platinum Taq DNA polymerase kit

Table 1 Properties of the one-step multiplex RT-PCR primers

Primer name	Sequence 5′–3′	Product size (bp)	Position	Acc. no.	Reference
CEV-R	CCGGGGATCCCTGAAGGACTT	371	78–98	NC-001464	Gross et al. (1982)
CEV-F	GGAAACCTGGAGGAAGTCGAG		99–119		
CBLVd-R	TTCGTCGACGACGACCAGTC	234	86–104	M74065	Ashulin et al. (1991)
CBLVd-F	CCCTTCACCCGAGCGCTGCTT		188–208		
HSVd-R	CCGGGGCTCCTTTCTCAGGTAAGT	302	59–82	NC-001351	Sano et al. (1988)
HSVd-F	GGCAACTCTTCTCAGAATCCAGC		83–105		
CVd-III-R	CGTCACCAACTTAGCTGCCTTCGT	269	91–112	NC-003264	Sieburth et al. (2002)
CVd-III-F	GTCTCCGCTAGTCGGAAAGACTCCG		135–159		
CVd-IV-R	CCGGGGATCCCTCTTCAGGT	138	52–71	X14638	Puchta et al. (1991)
CVd-IV-F	GGTGGATACAACTCTTGGGTTGT		217–239		
UBQ[a]-R	GTTGATTTTTGCTGGGAAGC	194	571–590	M74156.1	Amy et al. (2004)
UBQ-F	GATCTTCGCCTTAACGTTGT		746–765		

R antisense primer, *F* sense primer

[a] Internal control

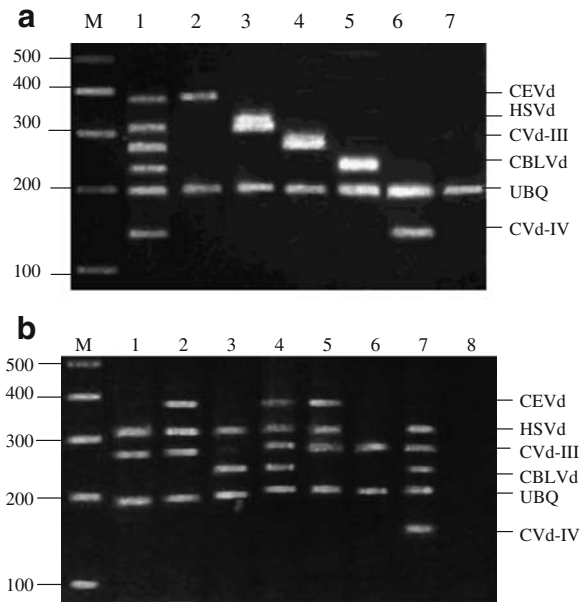

Fig. 1 **a** One-step multiplex RT-PCR analysis of single infection extracts and their mixture. *Lane 1* mix of total nucleic acids tested in lanes 2–6; *lane 2* CEVd; *lane 3* HSVd; *lane 4* CVd-III; *lane 5* CBLVd; *lane 6* CVd-IV; *lane 7* healthy control. **b** Multiplex RT-PCR analysis of infected citrus trees in field (lanes 1–6 correspond to six samples listed in Table 2 respectively). *Lane 1* No. 3; *lane 2* No. 5; *lane 3* No. 29; *lane 4* No. 8; *lane 5* No. 1; *lane 6* No. 21; *lane 7* Satsuma mandarin from Turkey; *lane 8* water control. *M* 100 bp molecular marker

(Invitrogen). The reaction was performed with the mixture of the all viroid primer pairs at a final concentration of 0.2 μM except primers of CEVd (0.5 μM), HSVd (0.1 μM). Primers to the *Ubiquitin* gene were used as an internal control (UBQ-F, -R, each at 0.15 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; 0.4 μl 50 mM $MgSO_4$; 0.25 μl 10 mM dNTPs; 0.4 μl 100 mM DTT; 2 μl of cocktail primers; 0.4 μl of RT-Taq; 0.55 μL of sterile water. Optimal one-step multiplex RT-PCR parameters were an initial incubation at 50°C for 30 min for cDNA synthesis, followed by denaturation of the RT enzyme for 2 min at 94°C and then, 35 cycles of denaturation at 94°C for 30 s, primer annealing at 58°C for 30 s and primer extension at 68°C for 45 s, followed by final extension at 68°C for 7 min. The amplified PCR products were analysed on 2% agarose gels stained with ethidium bromide.

To confirm the specificity of the assay, PCR products were purified and ligated into the pGEM T-easy vector (Promega) and recombinant plasmids from transformed cells were subjected to sequence analysis. In all cases, the DNA sequences were obtained as expected for each viroid (data not shown). Though *18S* rRNA is frequently used as an internal control, it is far from ideal because it is not always expressed at a constant level in all conditions. The *Ubiquitin* gene, which has a higher expression stability and expression level than most of house-keeping genes, was selected for an internal control in this study (Amy et al. 2004). Figure 1a shows the amplification results for single infection extracts, individually or combined, in the multiplex RT-PCR system. No significant primer-dimer and false-positives were observed in any of the samples tested. The expected product for the internal control was detected in all citrus trees analysed. The six expected products were detected when the mixture of five single infection extracts was performed by multiplex RT-PCR (Fig. 1a, lane 1). Figure 1b shows the amplified products by one-step multiplex RT-PCR from extracts of seven infected samples and a water control. Most of the infected citrus plants harboured more than one viroid species, and a few plants were infected with up to four citrus viroids (Fig. 1b, lane 4: Zhitong navel orange from Sichuan province; lane 7: Satsuma mandarin from Turkey and Fig. 2).

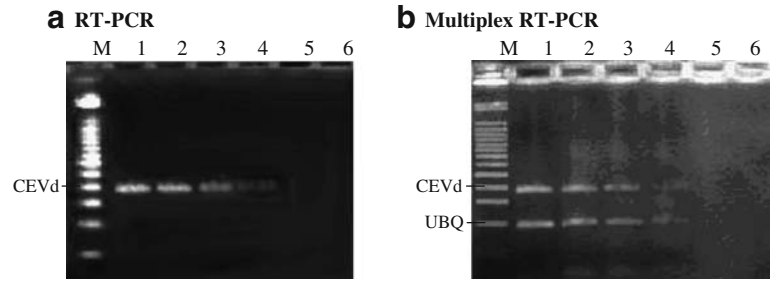

Fig. 2 Comparison of the respective sensitivities of RT-PCR and multiplex RT-PCR for the detection of CEVd. *Lane 1–5* ten-fold serial dilutions of a CEVd positive control respectively; *lane 6* water control; *M* 100 bp molecular marker

Table 2 One-step multiplex RT-PCR assays of 40 viroid-infected field samples from sweet oranges, mandarins, mandarin hybrids and lemons in China

No.	Cultivar	Origin of samples	Multiplex RT-PCR				
			CEV	CBLVd	HSVd	CVd-III	CVd-IV
1	Fengyuan No. 72-1 navel orange	Chongqing	+	−	+	+	−
2	Fengyuan No. 95-1 navel orange	Chongqing	+	−	+	+	−
3	Fengyuan No. 91 navel orange	Chongqing	−	−	+	+	−
4	Skaggs Bonaza navel orange	Chongqing	−	−	+	+	−
5	Shirayanagi navel orange	Chongqing	+	−	+	+	−
6	Shirayanagi navel orange	Chongqing	−	−	+	+	−
7	Meishan No.9 navel orange	Sichuan	+	−	+	−	−
8	Zhitong navel orange	Sichuan	+	+	+	+	−
9	Changning No. 4 navel orange	Sichuan	+	−	+	+	−
10	Newhall navel orange	Chongqing	−	+	+	+	−
11	Newhall navel orange	Guizhou	−	−	+	+	−
12	Newhall navel orange	Jiangxi	+	−	+	+	−
13	Skaggs Bonaza navel orange	Jiangxi	+	−	+	+	−
14	Yoshida navel orange	Jiangxi	−	−	+	+	−
15	Seike navel orange	Guangxi	−	−	+	+	−
16	Anliucheng sweet orange	Guangxi	−	−	+	+	−
17	Anliucheng sweet orange	Guangxi	−	+	−	−	−
18	Anliucheng sweet orange	Guangdong	−	+	+	+	−
19	Xinhuicheng sweet orange	Guangdong	+	−	+	+	−
20	Hamlin sweet orange	Chongqing	−	+	+	+	−
21	Zhongyu No.7 sweet orange	Chongqing	−	−	−	+	−
22	Beibei 447 jincheng	Chongqing	−	+	+	−	−
23	Valencia orange	Chongqing	+	+	+	+	−
24	Yiyuan No.73-6 jincheng	Sichuan	−	−	+	+	−
25	Yiyuan No.3 jincheng	Sichuan	−	−	+	+	−
26	Tarocco blood orange	Sichuan	+	−	+	+	−
27	Moroge Sanguine orange	Chongqing	+	−	+	+	−
28	Yamashitabeni wase unshu	Hunan	−	+	+	+	−
29	Nankan unshu No.20	Hunan	−	+	+	−	−
30	Fenglong tangor	Chongqing	+	−	+	−	−
31	Kiyomi tangor	Chongqing	+	−	+	+	−
32	Harumi tangor	Chongqing	−	+	+	−	−
33	Shiranuhi tangor	Chongqing	+	−	+	+	−
34	Shiranuhi tangor	Zhejiang	+	−	+	+	−
35	Harumi tangor	Zhejiang	−	+	+	+	−
36	Ehime Kashi No. 22	Zhejiang	−	−	+	+	−
37	Ehime Kashi No. 22	Hunan	−	+	+	+	−
38	Ehime Kashi No. 14	Hunan	−	−	+	+	−
39	Eureka lemon	Chongqing	+	−	+	+	−
40	Eureka lemon	Sichuan	+	−	+	−	−
Number of positive (%)			18 (45)	12 (30)	38 (95)	33 (82.5)	0 (0)

Serial dilution experiments showed that the uniplex RT-PCR sensitivity was similar to multiplex RT-PCR for all viroids. This is shown for CEVd (371 bp) which gives the lowest amplification level in the multiplex system in Fig. 2. No discrepancies were observed between the uniplex RT-PCR for each viroid using only the specific primer pair and multiplex RT-PCR procedure using a mixture of all primers in the same

reaction, demonstrating that interference problems did not occur in naturally infected samples from the field. The expected PCR fragments were observed until dilution 10−4 of the original total nucleic acid extracts.

Table 2 shows the results obtained from representative 40 viroid-infected citrus trees comprising of sweet orange (*C. sinensis*), lemon (*C. jambhiri*), mandarin (*C. reticulata*) and mandarin hybrid. The results obtained by one-step multiplex RT-PCR corresponded exactly to those from sPAGE and single RT-PCR for amplifying full-length sequence of each viroid (data not shown). The citrus samples of different varieties investigated in the study were from major citrus growing regions, therefore representing a wide geographical distribution and host diversity of citrus viroids. Twenty-five of the 40 samples were cultivars imported from abroad, and the remaining samples were all local cultivars. CEVd, HSVd and CVd-III were the predominant viroid species in the field. The distribution of CBLVd in China was sporadic, whereas so far CVd-IV was not detected in any of the samples collected. The most frequent viroid combinations were CEVd+HSVd+CVd-III and HSVd+CVd-III. As described by Duran-Vila et al. (1988), field-grown citrus trees often harbour complex mixtures of several different viroid species, and our results confirm the phenomenon.

Control of viroid spread is critical in nurseries where the contamination of a single mother tree may result in the dissemination of many infected plants, so it is essential to monitor mother trees periodically. This rapid and specific one-step multiplex RT-PCR assay provides an effective and reliable molecular diagnostic tool for the detection and identification of citrus viroids.

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