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Development of a multiplex RT-PCR detection and identification system for *Potato spindle tuber viroid* and *Tomato chlorotic dwarf viroid*

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Abstract *Potato spindle tuber viroid* (PSTVd) and *Tomato chlorotic dwarf viroid* (TCDVd) are two closely related Pospiviroids which cause economically important diseases on tomato (*Solanum lycopersicum*). Until now, however, there have been no molecular diagnostic methods available for discriminating between them except sequencing. We have developed a multiplex reverse transcription-polymerase chain reaction (RT-PCR) system that simultaneously detects and discriminates between both viroids in one reaction. Using this system, amplified cDNAs resulted in a 271 bp PCR product when PSTVd is detected as the template and 191 bp when TCDVd is detected. This multiplex RT-PCR system was used to accurately detect both viroids in field cultivated tomato and petunia (*Petunia* × *hybrida*) plants. This is the first finding of PSTVd in field grown tomatoes in Japan.

Keywords Multiplex PCR · Diagnosis · Tomato · Potato · Petunia · PSTVd · TCDVd

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Viroids are the smallest and simplest of the plant pathogens, consisting of a single-stranded circular naked RNA genome, 246–400 nucleotides in length, which lack any protein coding sequences (Tabler and Tsagris 2004). Approximately 30 viroids have been found worldwide and are classified into two families: the *Pospiviroidae* and the *Avsunviroidae* (Tabler and Tsagris 2004). *Potato spindle tuber viroid* (PSTVd), which is the type viroid of the genus *Pospiviroid* and the family *Pospiviroidae,* has a wide host range of at least 138 species in ten families (Singh 1973). Natural infections of PSTVd have been found mainly in solanaceous crops, such as pepino (Puchta et al. 1990), potato (Diener and Raymer 1971), tomato (Puchta et al. 1990) and avocado (*Persea americana*, Querci et al. 1995) in the field so far. Also, it has recently been reported that a PSTVd caused disease in greenhouse tomatoes in New Zealand (Elliot et al. 2001), Australia (Mackie et al. 2002) and Belgium (Verhoeven et al. 2007a).

Tomato chlorotic dwarf viroid (TCDVd) also belongs to the genus *Pospiviroid*, and causes a disease on tomato plants characterized by leaf chlorosis, leaf yellowing and dwarfing, leading to reduction of fruit production. TCDVd was first discovered in tomatoes cultivated in Canada (Singh et al. 1999), and was subsequently reported on tomatoes (Verhoeven et al. 2004) and petunias (*Petunia* × *hybrida*) in the USA (Verhoeven et al. 2007b), *Verbena* × *hybrida* in India (Singh et al. 2006), tomatoes in Japan (Matsushita et al. 2008), petunias in the UK that had been imported

from Israel and Portugal (James et al. 2008), tomatoes in USA (Ling et al. 2009), tomatoes in Canada (Singh et al. 2010), and tomatoes in France (Candresse et al. 2010). Although TCDVd and PSTVd share 85–89% nucleotide sequence identity (Singh et al. 1999) and produce symptoms on a tomato plant which are indistinguishable, each viroid has a few different host plants. Whereas *Gomphrena globosa* is a host for PSTVd (Singh 1973; Singh and Bagnall 1968), plants were not susceptible to infection by TCDVd (Matsushita et al. 2009).

Reverse transcription-polymerase chain reaction (RT-PCR) has been widely used as a highly sensitive and specific detection method for infectious diseases, including viroids. Although RT-PCR for the detection of PSTVd and TCDVd using primer set 3P + 4P (Table 1, Fig. 2a) has been established by Behjatnia et al. (1996), it cannot distinguish between PSTVd and TCDVd in a single PCR (Fig. 2a). In order to identify either viroid, it is necessary to either carry out RT-PCR twice using separate reactions and specific primer sets for each viroid, or, confirm the sequence of cDNA products amplified by RT-PCR using specific primers. Multiplex RT-PCR has previously been described for the simultaneous detection of several other viroids (Hosokawa et al. 2007; Ito et al. 2002; Wang et al. 2009), providing a quick, reliable and cost-effective method for routine diagnosis. Therefore, we have developed a multiplex RT-PCR for the simultaneous detection of PSTVd and TCDVd.

Primers for multiplex RT-PCR were designed from the complete nucleotide sequences of PSTVd and TCDVd obtained from GenBank/EMBL/DDBJ (http://www.ddbj.nig.ac.jp/). Sequence alignments were performed using the CLUSTAL W program (Thompson et al. 1994). A multiplex reverse primer (MpR primer) for the first-strand synthesis was designed from a highly conserved region between PSTVd and TCDVd (Table 1). To specifically detect PSTVd but not TCDVd, multiplex forward primers were selected at nucleotide range 126–141 of PSTVd, which is a unique region of the RNA sequence of this viroid. As it turns out, however, the PSTVd sequences registered in GenBank/EMBL/DDBJ have different nucleotides in positions 140 and 141, leading to the four following sequences: UA, UC, CU and CA. Accordingly, four forward primers, MpPTAF for UA, MpPTCF for UC, MpPCTF for CU and MpPCAF for CA, were designed for multiplex RT-PCR as shown in Table 1.

RNA sequences of PSTVd developed to serve as templates for multiplex RT-PCR were prepared from the cDNA clones by using an *in vitro* T7 RNA transcription system as follows. First, a cDNA template was amplified by RT-PCR with a primer set 3P + 4P (Behjatnia et al. 1996) using the mild

Table 1 Primer sequences used for the multiplex or standard RT-PCR method for TCDVd and PSTVd detection

Viroid	Primer name	Direction	Nucleotide sequence (5′-3′)	Position		Reference
				PSTVd	TCDVd	
PSTVd, TCDVd	3P	Reverse	CCGGATCCCTGAAGCGCTCCTCCGAGC	68–93	68–95	Behjatnia et al. (1996)
	4P	Forward	TCGGATCCCCGGGGAAACCTGGAGCG	87–110	84–111	
PSTVd	1P	Reverse	GTTTCCACCGGGTAGTAG	254–271		
	2P	Forward	AACTGAAGCTCCCGAGAACCGC	272–293		
TCDVd	T2R	Reverse	CTGTTTCGCCTTCCACAAG		135–153	This study
	T1F	Forward	ACAACTGAAGCTCCCGAGAA		272–291	
Multiplex	MpR	Reverse	TCAGGTGTGAACCACAGGAA	18–37	18–37	
	MpTF	Forward	CTTCCTTTGCGCGCCACT		206–223	
	MpPTAF	Forward	CGGTGGGGAGTGCC**TA**	126–141		
	MpPTCF	Forward	CGGTGGGGAGTGCC**TC**	126–141		
	MpPCTF	Forward	CGGTGGGGAGTGCC**CT**	126–141		
	MpPCAF	Forward	CGGTGGGGAGTGCC**CA**	126–141		

The different sequence between forward primers, MpPTAF, MpPTCF, MpPCTF and MpPCAF, for PSTVd detection in multiplex are depicted in gothic

isolate of PSTVd (GenBank/EMBL/DDBJ accession no. X76844; Gora et al. 1994) that has CA in the position 140 and 141. Next, this cDNA template was ligated between a T7 and a SP6 promoter in the pGEM-T Easy vector (Promega, Madison, WI, USA) to form a base cDNA clone, named pPSV-CA, used to make the other three nucleotide changes. This clone covers the full-length nucleotide sequence of the parental isolate, but is not infectious. Then, the other three nucleotide changes in the position 140 and141 were prepared by a PCR-based mutagenesis method using a KOD plus mutagenesis kit (Toyobo, Osaka, Japan). These three cDNA clones were labelled pPSV-TA for UA, pPSV-TC for UC and pPSV-CT for CU, respectively. Lastly, RNA sequences of each PSTVd cDNA clones served as templates for multiplex RT-PCR, which were synthesized from each plasmid with a T7 RiboMax Large Scale RNA kit (Promega, Valencia, CA, USA).

Although a mixture of four forward primers and a reverse MpR primer has been tested in a standard RT-PCR method by Matsushita et al. (2008), when we attempted this procedure, many non-specific and smeared bands appeared in the gel (data not shown). We discovered that when using a mixture of three forward primers, excluding MpPCAF, in the RT-PCR we were able to sufficiently amplify a specific PCR band of 271 bp from all four sequences of PSTVd including the CA variation (Fig. 1) without a cross reaction to TCDVd and non-specific smearing (data not shown). Hence, three forward primers, MpPTAF, MpPTCF and MpPCTF, and the MpR reverse primer were chosen for development of the multiplex RT-PCR system.

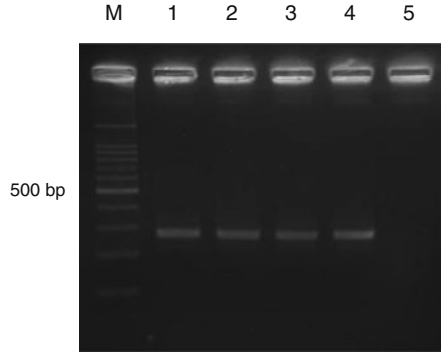
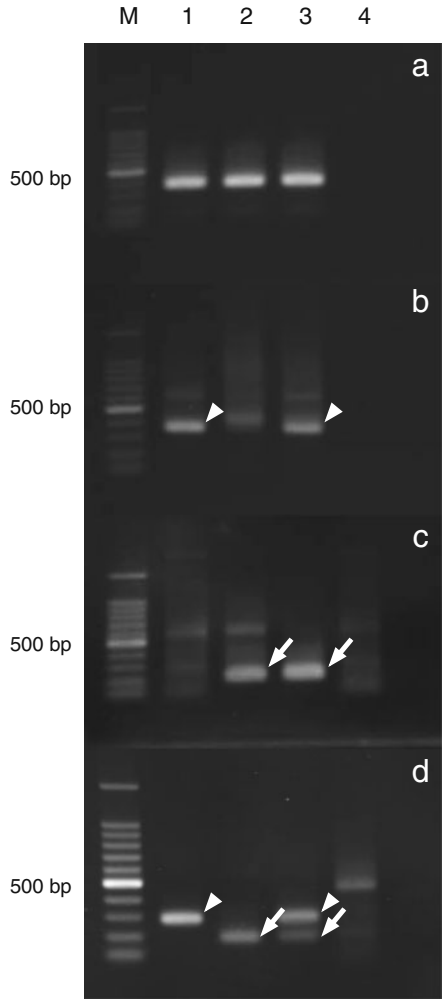


Fig. 1 Products generated by the RT-PCR with a primer cocktail of three forward primers, MpPTAF and MpPTCF, MpPCTF, and a reverse primer, MpR, to detect all four types of PSTVd. Lane 1: PSTVd-UA RNA. Lane 2: PSTVd-UC RNA. Lane 3: PSTVd-CU RNA. Lane 4: PSTVd-CA RNA. Lane 5: RNase-free water. Lane M: 100-bp ladder markers (TaKaRa, Otsu, Japan)

To simultaneously detect TCDVd and PSTVd in the same multiplex RT-PCR, we chose a forward primer, MpTF, in a unique and highly conserved region of all TCDVd isolates registered in GenBank/EMBL/DDBJ, based on the alignment analysis using the CLUSTAL W program, to generate a PCR band of different size from that of PSTVd in the system. These primers generate a PCR band of 191 bp when TCDVd is detected, whereas the band generated when PSTVd is detected is 271 bp as shown in Fig. 1. The two bands representing each viroid are easily resolved by a 1.5% agarose gel electrophoresis. Based on these results we finally chose: MpPTAF, MpPTCF and MpPCTF as forward primers for PSTVd; one forward primer, MpTF, for TCDVd, combined in a forward primer cocktail; and one reverse primer, MpR, for both viroids in the multiplex RT-PCR system.

In developing the multiplex system, multiplex RT-PCR was first compared to the standard RT-PCR method previously described (Matsushita et al. 2008). First, enough of the template consisting of the RT product was made for both the standard and the multiplex PCR. To prepare the RT reaction, 1 μl of extracted RNA was added to 9 μl of RT mixture. The RT mixture contained 2 μl of RT buffer, 1 μl of dNTPs (10 mM), 0.5 μl of reverse primer solution (20 μM), 0.5 μl of RNase inhibitor (1 U, Toyobo), 0.5 μl of reverse transcriptase (20 μM), and was brought up to a final volume of 9 μl with distilled water. The mixture was incubated at 42°C for 30 min followed by 99°C for 5 min. After the RT reaction, 1.6 μl or 1 μl of RT product was added to 8.4 μl of the standard PCR mixture or 9 μl of the multiplex PCR mixture, respectively. The standard PCR mixture consisted of 0.1 μl of forward primer solution (20 μM), 1 μl of reaction buffer, 0.1 μl of KOD Dash polymerase (Toyobo), 1 μl of dNTPs (2 mM), and was brought up to a final volume of 8.4 μl with distilled water. The multiplex PCR mixture consisted of 1 μl of dNTPs (2 mM), 0.4 μl of forward primer cocktail (MpPTAF, MpPTCF, MpPCTF and MpTF; 10 μM each), 1 μl of KOD Dash polymerase, and was brought up to a final volume of 9 μl with distilled water. The standard and multiplex PCR conditions were 35 cycles of 45 s of melting at 98°C, 10 s of annealing at 62°C, and 45 s of extension at 74°C.



◀ **Fig. 2** Products generated by standard RT-PCR (**a-c**) and multiplex RT-PCR (**d**) methods for PSTVd and/or TCDVd detection. **a**: 3P and 4P primers. **b**: 1P and 2P primers. **c**: T1F and T2R primers. **d**: MpR for the RT and reverse primer, and the forward primer cocktail (MpPTAF, MpPTCF, MpPCTF and MpTF; see in text). Lane 1: PSTVd-infected tomato. Lane 2: TCDVd-infected tomato. Lane 3: mixture of above two tomatoes. Lane 4: mock-inoculated tomato. Lane M: 100-bp ladder markers (TaKaRa, Otsu, Japan). Arrowheads indicate PSTVd bands and arrows are TCDVd bands

For use as a template for RT-PCR, total RNA was extracted from 100 mg frozen leaves of a tomato plant infected with either PSTVd (Accession no. X76844; Gora et al. 1994) or TCDVd (Accession no. AB329668; Matsushita et al. 2008) by using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Both total RNA extracts were equally mixed and tested by multiplex RT-PCR compared to standard RT-PCR using the primer sets of 3P + 4P (Behjatnia et al. 1996), 1P + 2P (Behjatnia et al. 1996) and T1F + T2R (unpublished) (Table 1). The RT-PCR products (10 μl) were separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

When the total RNA extracted from tomato plants infected with PSTVd and TCDVd were assessed with the multiplex primer set (Table 1), cDNA fragments of size 271 for PSTVd and 191 bp for TCDVd were obtained. Those fragments corresponded (Fig. 2d, lane 1, 2) with the identical cDNA sequence for each viroid. Also, when transcripts from pPSV-TA, -TC, -CT and -CA with TCDVd RNA were used as templates in a multiplex RT-PCR, the same two bands were detected. This demonstrates that all variants of PSTVd (PSTVd-UA, -UC, -CU and -CA) in the nucleotide position 140 and 141 can be detected by this multiplex RT-PCR (data not shown). However, one uncertain feature still remains in this detection system even though four types of PSTVd variants have experimentally been covered. PSTVd-N has been previously described as a strange isolate exhibiting major sequence differences to all other PSTVd isolates (Puchta et al. 1990). When aligned the sequence of the appropriate forward primer (MpPTCF; 5′-CGGTGGGGAGTGCCTC-3′) with the correspondent region of PSTVd-N, two nucleotides between 126 and 141 were missing that were in 136 and 137 (126–141, 5′-CGGTGGGGAG__CCTC-3′; Accession no. X17268) but the position of 140 and 141 was same. Nevertheless, it is suspected to be able to detect PSTVd-N by this multiplex RT-PCR

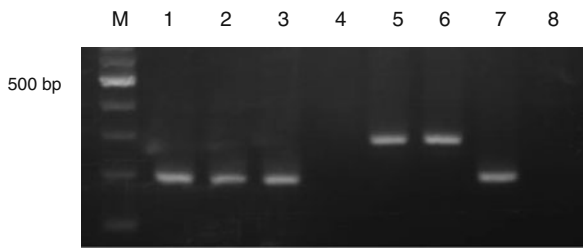


Fig. 3 Detection of TCDVd and PSTVd from field samples using the multiplex RT-PCR system. Lane 1: tomato from Hiroshima Prefecture. Lane 2: tomato from Chiba Prefecture. Lane 3: petunia from Chiba Prefecture. Lane 4: tomato from Tochigi prefecture. Lane 5: tomato from Fukushima Prefecture. Lane 6: PSTVd-infected tomato as a positive control. Lane 7: TCDVd-infected tomato as a positive control. Lane 8: mock-inoculated tomato. Lane M: 100-bp ladder markers (TaKaRa, Otsu, Japan)

system because the four nucleotides of the 3′ end of the MpPTCF primer are identical with that of PSTVd-N. In addition, no cDNA fragments were obtained from healthy tomato plants (Fig. 2d, lane 4). When using the primers listed in Table 1, the 3P + 4P primer pair amplified a single band irrespective of whether the sample was infected with PSTVd and/or TCDVd (Fig. 2a. lane 1, 2). The 1P + 2P primer pair amplified PSTVd, but not TCDVd (Fig. 2b. lane 1, 2), whereas T1F + T2F amplified TCDVd but not PSTVd (Fig. 2c. lane 1, 2). When mixed total RNA extracts of both viroids were tested using multiplex RT-PCR, cDNA fragments from each of the viroids were detected (Fig. 2d, lane 3).

The field performance of the multiplex RT-PCR system was assessed by testing tomato and petunia plants cultivated in different fields in the Hiroshima, Chiba, Fukushima, and Tochigi Prefectures in Japan. Total RNA was prepared as previously described (Matsushita et al. 2008). TCDVd was detected in tomato plants from Hiroshima (Fig. 3, lane 1) and Chiba Prefectures, and in petunia from Chiba Prefecture (Fig. 3, lane 2, 3). Unexpectedly, PSTVd was found in 2 samples collected from tomato plants cultivated in Fukushima Prefecture during 2008 (Fig. 3, lane 5). To confirm the complete sequence of PSTVd, full-length PCR products were amplified by RT-PCR using the 3P + 4P primer pair (Table 1), then cloned into pGEM-T Easy vector (Promega). The nucleotide sequence of the 359 bp product obtained from both of suspected tomato plants was homologous to the submitted sequence of PSTVd (Accession no. EU862231; Verhoeven et al. 2009). This is the first finding of PSTVd-infecting tomato plants present in a cultivated field, domestically.

In conclusion, these results show that the multiplex RT-PCR system developed, using the primers specified, is useful for detection of PSTVd and TCDVd in a single reaction in field surveys and quarantine testing.

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