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Multiplex reverse transcription loop-mediated isothermal amplification for the simultaneous detection of CVB and CSVd in chrysanthemum

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A multiplex reverse transcription loop-mediated isothermal amplification (mRT-LAMP) assay was developed for the simultaneous detection of *Chrysanthemum Virus B* (CVB) and *Chrysanthemum stunt viroid* (CSVd), which are the major viral pathogens of chrysanthemum worldwide. Two sets of mRT-LAMP primers were designed for the coat protein gene of CVB and the complete nucleotide sequence of CSVd, and a restriction enzyme cleavage site was inserted into two pairs of species-specific primers. The mRT-LAMP assay was designed by combining these two sets for a total of eight primers. The mRT-LAMP method distinguished between CVB and CSVd due to the subsequent restriction enzyme analysis. The sensitivity of the mRT-LAMP method was 10^3 times higher than classical PCR regarding the detection limits for CVB and CSVd. No positive results were observed when RNA from other chrysanthemum pathogens were used as mRT-LAMP templates. The method was verified by testing chrysanthemum samples collected from Beijing and Henan Province and showed high reliability and sensitivity. The developed mRT-LAMP assay also offers an efficient, convenient, and rapid tool for screening chrysanthemum virus and viroid, especially CVB and CSVd, and can be diagnosed in a single reaction. These results suggest that the new mRT-LAMP method may be used routinely for virus and viroid surveys.

1. Introduction

Chrysanthemum (*Chrysanthemum morifolium*) is a commercially important ornamental plant worldwide and also has important value for edible and medicinal use. Traditionally, chrysanthemums are propagated mainly through cutting, which is highly conducive to the accumulation and spread of viral diseases. Viral disease causes chrysanthemum germplasm degeneration, which has become a prominent issue in chrysanthemum production. Currently, more than 20 types of viral pathogens that infect chrysanthemums have been reported (Bouwen and Zaayen, 1995; Verhoeven et al., 1996; Chen et al., 1999; Megan et al., 2001; Farzadfar et al., 2005; Toru et al., 2011).

In China, six viruses and two viroids have been reported in chrysanthemum, including *Chrysanthemum virus B* (CVB), *Tomato aspermy virus* (TAV), *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV), *Potato virus Y* (PVY), Potato virus X, *Chrysanthemum stunt viroid* (CSVd), and *Chrysanthemum chlorotic mottle viroid* (CChMVd) (Wu et al., 2002; Zhang et al., 2011a,b). CVB, a member of the genus Carlavirus, is a single-stranded RNA virus and the causal agent of a severe disease in chrysanthemum. Chrysanthemums infected with CVB have various symptoms from mild leaf mottling or vein clearing to severe mosaic or flower malformation; however, sometimes no symptoms are seen (Hollings, 1957; Hollings and Stone, 1972). CSVd, which belongs to the family Pospiviroidae (Palukaitis and Symons, 1980), has a single-stranded circular RNA genome 354 or 356 nucleotides in length that causes infection in chrysanthemums (Hollings and Stone, 1973; Gross et al., 1982). Chrysanthemums infected with CSVd have general symptoms, such as stunted growth, reduced flower size, and bleached blooms, which decrease the quality and yield of cut flowers (Sugiura and Hanada, 1998; Doi and Kato, 2004; Matsushita et al., 2007). CVB and CSVd can infect many chrysanthemum cultivars (Singh et al., 2007; Randles, 2004). Mixed viral infection is very common in

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Table 1
Viruses and viroids isolates from chrysanthemum in China used in this work.

Viruses or viroids	Designation	Origin
Chrysanthemum virus B	CVB	Beijing and Kaifeng Henan province,
Chrysanthemum stunt viroid	CSVd	Beijing and Kaifeng Henan province,
Tomato aspermy virus	TAV	Beijing
Chrysanthemum chlorotic mottle viroid	CChMVd	Chengdu Sichuan province
Tobacco mosaic virus	TMV	Chengdu Sichuan province
Cucumber mosaic virus	CMV	Kunming Yunnan province
Potato virus Y	PVY	Kunming Yunnan province

chrysanthemum fields. The complex infection of several viruses and viroids has caused severe losses in chrysanthemum commercial production in China and worldwide and is a crucial problem that needs to be solved.

The traditional methods for detecting CVB and CSVd include indicator plants, electron microscopy, serodiagnostic methods (Hakkaart et al., 1962; Raizada et al., 1989), DAS–enzyme-linked immunosorbent assays (Verma et al., 2003; Huttinga et al., 1987), RT-PCR, nested PCR (Verhoeven et al., 1998; Munetaka et al., 2004; Ohkawa et al., 2007; Atsushi et al., 2008), multiplex RT-PCR (Yan et al., 2009; Song et al., 2012), and single reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Shiro et al., 2005). However, these methods are laborious, time consuming, and require expensive equipment, and they are not likely to be rapid, sensitive, and appropriate for virus detection in the field. Also, they cannot detect different types of viruses and viroids simultaneously. The traditional single RT-LAMP method requires only one enzyme, and the amplification reaction proceeds under isothermic conditions (Notomi et al., 2000; Nagamine et al., 2002). Single RT-LAMP has high specificity because of the use of four primers that recognize six distinct regions on the target, and it usually results in high amplification efficiency in a very short time. Single RT-LAMP produces a large number of amplified products, making simple detection possible (Mori et al., 2004). The multiplex RT-LAMP (mRT-LAMP) method is based on single RT-LAMP and was developed for simultaneous detection of multiple pathogens in a single tube. However, mRT-LAMP detection is difficult because LAMP amplicons have complicated structures (Liang et al., 2012). Currently, mRT-LAMP is used only for simultaneous detection of white spot syndrome virus and infectious hypodermal and hematopoietic necrosis virus in penaeid shrimp (He and Xu, 2011).

In this work, mRT-LAMP for rapid simultaneous detection of CVB and CSVd in chrysanthemum is described. Using this method, identification of the two chrysanthemum pathogens in the same RT-LAMP reaction was possible. This method greatly improved the efficiency for rapid detection of the two chrysanthemum pathogens and can be used as a simple detection method for early warning and control of CVB and CSVd.

2. Materials and methods

2.1. Plant materials and virus (viroid) sources

Five chrysanthemum pathogenic viruses and two chrysanthemum pathogenic viroids were used in this work. Their names and origins are given in Table 1. The viruses and viroids TAV, CChMVd, TMV, CMV, and PVY were used to evaluate the specificity of mRT-LAMP. All samples were stored at −80 °C for later use.

2.2. Total RNA extraction

Total RNA was extracted from 100 mg chrysanthemum leaves using RNAiso Plus (TaKaRa, Dalian, China) according to the manufacturer's protocol. The RNA extracts were used to detect CVB, CSVd, TAV, CChMVd, TMV, CMV, and PVY with RT-PCR, and the confirmed samples were used in further experiments.

2.3. Conventional RT-PCR detection of CVB and CSVd

Total RNA from CVB- and CSVd-infected plants was used as a template for synthesis of first-strand cDNA. The first-strand cDNAs of CVB and CSVd RNAs were synthesized using M-MLV reverse transcriptase according to the manufacturer's instructions (Promega, Madison, WI, USA). The RT mixture included 2 μl extracted RNA, 2 μl dNTP mixture (2.5 mM), 0.5 μl (200 U) M-MLV reverse transcriptase, 2 μl M-MLV 5× reaction buffer, 0.5 μl (40 U) recombinant RNasin ribonuclease inhibitor, 0.5 μl random primers (10 μM), and sterile distilled water in a final volume of 10 μl. The mixture was incubated at room temperature (25 °C) for 10 min and then at 42 °C for 60 min. After the RT reaction, a 2-μl aliquot of the cDNA product was added to 18 μl of the PCR mixture, which consisted of 10 μl Taq Mastermix (Cwbio, Beijing, China), 2 μl forward and reverse primers (10 μM each), and 6 μl sterile distilled water (Table 2). PCR was conducted using an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 48 or 50 °C (CVB, 48 °C; CSVd, 50 °C) for 30 s, and 72 °C for 1 min. A final elongation step was performed at 72 °C for 10 min. The reaction products were analyzed by electrophoresis on 2.5% agarose gels. Molecular sizes of the amplified fragments were determined by comparison with DL2000 (TaKaRa) as a molecular weight marker.

2.4. Design of pathogen-specific primers for mRT-LAMP

The coat protein gene of CVB and the complete genome sequence of CSVd were sequenced and selected as reference sequences for primer design (Accession Nos. AJ585514 and DQ094298). mRT-LAMP requires a set of primers: a forward inner primer (FIP), a backward inner primer (BIP), and two outer primers (F3 and B3) (Notomi et al., 2000). The LAMP primers were designed using Primer Explorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html). For mRT-LAMP,

Table 2
Primers designed for detection of CVB and CSVd from chrysanthemum by RT-PCR.

Target virus	Primer name	Sequence (5′–3′)[a]	Tm (°C)	Size of PCR product (bp)
CVB	CVB-F	GCACCAGGTGATDMTGAARG	48	634
	CVB-R	GCATGTARTTCCAHGCHACAGC		
CSVd	CSVd-F	CCTGGAGAGGTCTTCTG	50	354
	CSVd-R	AGAGGAAGGAAACTAAAGGA		

[a] D = A/G/T; M = A/C; R = A/G; H = A/C/T.