International Journal of Agricultural Technology 2022Vol. 18(2):857-870
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Simultaneous and sensitive detection of CVB, CChMVd and CSVd mixed infections in chrysanthemum using multiplex nested RT-PCR

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Supakitthanakorn, S., Vichittragoontavorn, K., Kunasakdakul, K. and Ruangwong, O. (2022). Simultaneous and Sensitive Detection of CVB, CChMVd and CSVd mixed infections in chrysanthemum using multiplex nested RT-PCR. International Journal of Agricultural Technology 18(2):857-870.

Abstract Chrysanthemum plants are susceptible to infect by viral and viroid diseases. Early detection of these pathogens helps to prevent disease spread. Multiplex RT-PCR can simultaneously detect many virus and viroid in a single reaction, and applying multiplex nested PCR can improve the sensitivity of the detection. Chrysanthemum leaves were collected from cultivation areas in Northern Thailand. Multiplex nested RT-PCR using specific outer and inner primer pairs for detection of *Chrysanthemum virus B* (CVB), *Chrysanthemum chlorotic mottle viroid* (CChMVd), and *Chrysanthemum stunt viroid* (CSVd) were used for simultaneous detection. Among 15 randomly selected samples, one sample showed co-infections of CVB and CChMVd, and two samples showed multiple infections of all pathogens. Furthermore, multiplex nested RT-PCR detected amplicons of CChMVd and CSVd that were not detected by the first-round PCR. Also, sequence analysis was used to confirm the correction of amplified fragments and revealed that all amplicons were more than 93% identical compared to corresponding sequences deposited in the GenBank. Therefore, multiplex nested RT-PCR can be used for routine detection and diagnosis of virus and viroid diseases. This is the first detection of mixed infections (disease complex) both virus and viroid in the chrysanthemum of Thailand.

Keywords: Chrysanthemum, Virus, Viroid, Multiplex nested PCR

Introduction

Chrysanthemum (*Chrysanthemum x morifolium*) is an important and highly economic cut flower. Chrysanthemum propagation is achieved by implantation of seedlings which is a convenient and quick method and can obtain many shoots. However, this method carries a high risk from the infected

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mother plant, especially virus and viroid diseases (Cho *et al*., 2013). Chrysanthemum is commonly infected by viruses and viroids in single and multiple infections (Song *et al*., 2012). The effects of virus- and viroid-infected chrysanthemum are severe, including stunting, reduced flower size, poor root development and flower color bleaching, resulting in a decline in the quality and yield of cut flowers. However, the effects on infected chrysanthemum vary according to the chrysanthemum cultivar and environmental conditions (Matsushita, 2013). Therefore, detection and diagnosis of viruses and viroids in chrysanthemum can prevent the spread of pathogens and ensure pathogen-free reproductive stocks (Zhao *et al*., 2015).

Many viruses have been reported to infect chrysanthemum; among them, *Chrysanthemum virus B* (CVB) was the most frequent chrysanthemum-infecting virus (Singh *et al*., 2007). CVB is a member of the genus Carlavirus in the family Betaflexiviridae, which has slightly flexuous rod-like particles approximately 685 nm in length (Singh *et al*., 2007). CVB is transmitted by sap-inoculation and has aphids as an insect vector (Ohkawa *et al*., 2008). Some of the chrysanthemum plants infected by CVB show mild mosaic, vein clearing, or malformed leaves and flowers; otherwise, some cultivars were symptomless (Ohkawa *et al*., 2008).

Chrysanthemum plants are infected by two viroids consisting of *Chrysanthemum chlorotic mottle viroid* (CChMVd) and *Chrysanthemum stunt viroid* (CSVd) belonging to the families Avsunviridae and Pospiviroidae, respectively (Flores *et al*., 2005). CChMVd is a member of the genus Pelamoviroid and is 398–401 nucleotides long (Cho *et al*., 2013). The transmission of CChMVd occurs by grafting and plant sap (Navarro and Flores 1997; Cho *et al*., 2013). CChMVd causes yellow-green mottling, chlorosis, necrosis, and stunting in chrysanthemum plants (Dimock *et al.*, 1971; Yamamoto and Sano, 2005). Another viroid is CSVd, which belongs to the genus Pospiviroid and the whole genome composed of 354 nucleotides (Flores *et al*., 2005). It causes stunting, reduced flower size, and petal bleaching, resulting in significant decreases in flower quality (Chung *et al*., 2005). CSVd is transmitted by contaminated agricultural equipment and is a seed- and pollen- transmitted in chrysanthemum (Chung and Pak, 2008).

To detect and diagnose virus and viroid diseases, molecular techniques, such as reverse transcription-polymerase chain reaction (RT-PCR) and nucleic acid hybridization, are commonly used (Ebata *et al*., 2019). However, chrysanthemum plants are usually infected by various viruses and viroids; and single or simplex RT-PCR may not be appropriate for the detection. So, multiplex RT-PCR that simultaneously detects many targets at the same time has been recommended (Zhao *et al*., 2015).

Multiplex RT-PCR was developed for rapid and simultaneous detection of CVB, *Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY), *Tomato aspermy virus* (TAV), *Tobacco mosaic virus* (TMV), CChMVd, and CSVd mixed infections in chrysanthemum (Song *et al*., 2012; Zhao *et al*., 2015). For improving the sensitivity and specificity of multiplex detection, multiplex nested RT-PCR was applied by Bertolini *et al*. (2002) that detected four plant-infecting viruses and one bacterium in a single closed tube. The larger fragments were amplified by the first-round PCR using an outer primer pair; afterward, the smaller ones were amplified by the second round (nested) using an inner primer pair (Jinxia *et al*., 2011). The multiplex nested PCR technique fully combined the high sensitivity and specificity of nested PCR and the simultaneous rapidness of the multiplex PCR (Anna *et al*., 2010).

This study aimed to detect the infection of CVB, CChMVd, and CSVd in both single and multiple infections in chrysanthemum plants by using the multiplex RT-PCR and increase the sensitivity of the detection by applying the multiplex nested RT-PCR technique.

Materials and methods

Sample collection, RNA extraction, and reverse transcription (RT)

Chrysanthemum leaves showing virus and viroid-like symptoms, such as mosaic, chlorosis, vein banding, malformation, or stunting, were collected from cultivation areas in Chiang Mai and Chiang Rai Provinces of Northern Thailand during 2019–2020. Total RNA was extracted from leaves using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The RT reaction was performed using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan) according to the manufacturer's instructions and the cDNA was kept at -20 ℃ until use.

PCR detection

The single PCR detection was performed using EconoTaq® PLUS & PLUS GREEN 2× Master Mixes (Lucigen, USA). A total of 50 µl of PCR cocktail contained 25 µl of PCR master mix, 1 µl (10 µM) of outer primer pairs specific to the individual pathogen (Table 1), 1 µl of target DNA and DEPC water was added to make a final volume of 50 µl. The first-round PCR reactions were set at 94 ℃ for 4 min, 40 cycles at 94 ℃ for 30 s, 54 ℃ (CVB) or 58 ℃ (CSVd) or 59 ℃ (CChMVd) for 45 s and 72 ℃ for 45 s, followed by a final extension at 72 ℃ for 7 min. PCR reactions were conducted in a GeneMax

PCR Thermal Cycler (Bioer Technology, China). PCR products were visualized in 2.0% agarose gel electrophoresis staining with RedSafe™ Nucleic Acid Staining Solution (iNiTron, Korea).

The expected amplicons size of CVB, CChMVd, and CSVd were 621, 398, and 254 bp, respectively. For CSVd, the primer pairs included a primer pair for single and multiplex RT-PCR to amplify the partial genome (250 bp) and a primer pair for whole genome amplification (354 bp) that used for sequencing (Table 1).

Table 1. Primers used for multiplex RT-PCR and multiplex nested RT-PCR in this study

Virus/ viroid	Primer	Sequence (5'-3')	Product size (bp)	Ta[2] (℃)	Reference
CVB	PCR (Outer)	F[1]: AGTCACAATGCCTCCCAAAC R: CATACCTTTCTTAGAGTGCTATGCT	621	53	Guan *et al.* (2017)
	Nested PCR (Inner)	F: TCTGAAGGTGAGCCAAGCG R: CATATCCTCGGAAGTAGCCATG	381	57	
CChMVd	PCR (Outer)	F: CAGGATCGAAACCTCTTCCA R: GACCTCTTGGGGGTTAGAAA	398	59	Ebata *et al.* (2019)
	Nested PCR (Inner)	F: AGCGAAAGCTCTCTCCACAG R: ATCTGCCTAGGTTCCCCAGT	254	50	
CSVd	PCR (Outer)	F: CAACTGAAGCTTCAACGCCTT R: AGGATTACTCCTGTCTCGCA	250	58	Hosokawa *et al.* (2004)
	Nested PCR (Inner)	F: CCTATCTTCTTTAGCACCG R: AGTGGGGTCCTAAGCCCCAG	200	62	
	PCR (Whole genome)	F: TTCTTTCAAAGCAGCAGGGT R: AAAGAAATGAGGCGAAGAAG	354	54	Chung *et al.* (2005)

[1]F: forward primer and R: reverse primer, [2]Ta = annealing temperature

Nested PCR

An aliquot (1 µl) of the PCR product from the first-round PCR was used as the template for nested PCR amplification. The PCR cocktail was prepared as for a typical PCR assay, but the inner primer pairs were added instead of outer primer pairs (Table 1). The second-round PCR was performed at 96 ℃ for 5 min, 35 cycles at 96 ℃ for 30 s, 50 ℃ (CChMVd) or 57 ℃ (CVB) or 62 ℃ (CSVd) for 1 min, and 72 ℃ for 30 s, followed by a final extension at 72 ℃ for 7 min. PCR products were analyzed by agarose gel electrophoresis.

Multiplex PCR and multiplex nested PCR

Multiplex PCR cocktail was prepared as of single PCR reaction, and all outer primer pairs for CVB, CChMVd, and CSVd were added in the same tube at the volume of 1 µl each (10 uM). DNA from positive PCR detection of individual pathogens or naturally mixed infections was used as a template. The first-round PCR was performed at 96℃ for 5 min, 40 cycles at 96℃ for 30 s, 56℃ for 45 s, and 72℃ for 45 s, followed by a final extension at 72℃ for 7 min. PCR products were visualized by agarose gel electrophoresis. The second-round multiplex PCR, 1 µl PCR products of first-round multiplex PCR was aliquot to use as a DNA template, and the PCR cocktail was prepared as described for multiplex PCR detection, but inner primer pairs were used instead of outer primer pairs to amplify the shorter fragments (Table 1). The second-round multiplex PCR was performed at 96℃ for 5 min, 35 cycles at 96℃ for 30 s, 53℃ for 1 min, and 72℃ for 30 s, followed by a final extension at 72℃ for 7 min. PCR products were visualized by agarose gel electrophoresis.

Sensitivity test of multiplex RT-PCR and multiplex nested RT-PCR

To determine the detection limits of the multiplex RT-PCR and multiplex nested RT-PCR assays. The cDNA was serially ten-fold diluted (10^{0}–10^{-10}) and used as the template for multiplex RT-PCR amplification using both outer and inner primer pairs. For multiplex nested RT-PCR assay, the PCR product from first-round PCR was also serially ten-fold diluted, and the amplification reactions were prepared and conducted as described above.

Specificity test of multiplex RT-PCR and multiplex nested RT-PCR

To determine the possibility of cross reaction of multiplex RT-PCR and multiplex nested RT-PCR in detection of CChMVd, CSVd and CVB. Other viruses that were reported to infect chrysanthemum consisting of *Tobacco mosaic virus* (TMV), a member in genus *Tobamovirus*, and *Turnip mosaic virus* (TuMV), a member in genus *Potyvirus*, and viruses from other genera including *Papaya ringspot virus* (PRSV), a member in genus *Potyvirus*, and *Melon yellow spot virus* (MYSV), a member in genus *Orthotospovirus*, were selected as subjects in the specificity assay. The PCR mixture and conditions were prepared and set as described above.

Application of multiplex nested RT-PCR in actual samples

The 16 ramdom chrysanthemum samples were selected, based on their previous detection results that were positive to virus and viroid infection

detected by RT-PCR and other samples showed symptomless, to test the efficiency of multiplex RT-PCR and multiplex nested RT-PCR.

Sequence analysis

PCR products of both PCR and nested PCR were purified using PCR clean-up and gel extraction kits (Bio-Helix, Taiwan) according to the manufacturer's instructions. Nucleotide sequences were directly analyzed from purified PCR products in the fluorescent dye-terminator sequencing on ABI Prism™ 3730xl DNA sequencers (Applied Biosystems, Foster City, CA). All obtained sequences were aligned and analyzed and aligned using BLAST analysis and Molecular Evolutionary Genetics Analysis (MEGA) version X program (Kumar *et al.,* 2018), respectively. Only sequences obtained from the first round PCR would be deposited in GenBank to obtain accession numbers.

Results

RT-PCR and nested PCR detection

The first round of single RT-PCR detection showed that amplicons of CChMVd, CSVd, and CVB were detected with 398, 250, and 621 bp in size, respectively, while no products were detected from a negative control (Figure 1A). After the second round (nested), PCR amplicons of CChMVd, CSVd and CVB were 254, 200 and 381 bp respectively (Figure 1B). Also, the whole genome of CSVd was amplified with an amplicon of 354 bp (Figure 1C).

Multiplex PCR and multiplex nested PCR detection

Multiplex RT-PCR detection, using a DNA template from naturally mixed infections of 3 pathogens, showed that all amplicons of CChMVd (398 bp), CSVd (250 bp), and CVB (621 bp) were successfully amplified at the annealing temperature at 56℃ (Figure 1A). The multiplex nested RT-PCR reaction worked at the annealing temperature at 53℃ by simultaneously amplifying all amplicons of CChMVd (254 bp), CSVd (200 bp), and CVB (381 bp) (Figure 1C). Therefore, multiplex RT-PCR and multiplex nested RT-PCR detections were set as described conditions to detect virus and viroid mixed infections from 15 randomly selected chrysanthemum samples.

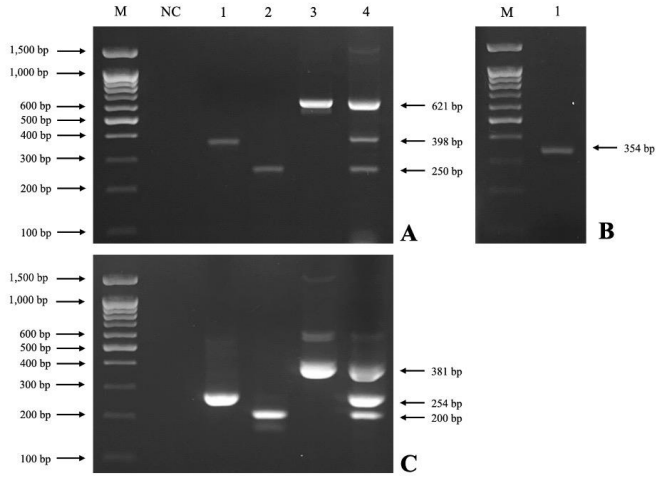

Figure 1. Detection of CVB, CChMVd and CSVd in chrysanthemum by multiplex RT-PCR and multiplex nested RT-PCR assays at 56℃ and 53℃ of annealing temperature, respectively. M: 100 bp + 1.5 kb DNA Ladder (SibEnzyme, Russia) and NC: negative control. (A) Multiplex RT-PCR detection (above); lane 1: 398 bp fragment of CChMVd; lane 2: 254 bp fragment of CSVd; lane 3: 621 bp fragment of CVB; lane 4: multiplex PCR detection of all pathogens obtained from first round PCR, (B) lane 1: 354 bp fragment of CSVd's whole genome and (C) multiplex nested RT-PCR detection; lane 1: 254 bp fragment of CChMVd; lane 2: 200 bp fragment of CSVd; lane 3: 381 bp fragment of CVB; lane 4: multiplex nested RT-PCR detection of all pathogens obtained from second round PCR

Sensitivity test

The sensitivity for detection limits of multiplex RT-PCR and multiplex nested RT-PCR assays were evaluated by serial ten-fold dilution of cDNA and PCR product from first-round PCR, respectively. For multiplex RT-PCR using outer primer pairs, virus and viroid-specific fragments for CChMVd, CSVd, and CVB were detected after diluting to an endpoint of 10^{-1} or called the limit of detection (LOD) was 10^{-1} (Figure 2A). In case of using inner primer paris for amplifying shorter fragments, the LOD were 10^{-2} that were 10 times higher than multiplex RT-PCR using outer primer pairs (Figure 2B). For multiplex nested RT-PCR, all bands were detected at dilution to the endpoint of 10^{-9} (Figure 2C). Therefore, the sensitivity of multiplex nested RT-PCR was at least 10^{7} times greater than the sensitivity of multiplex RT-PCR assays.

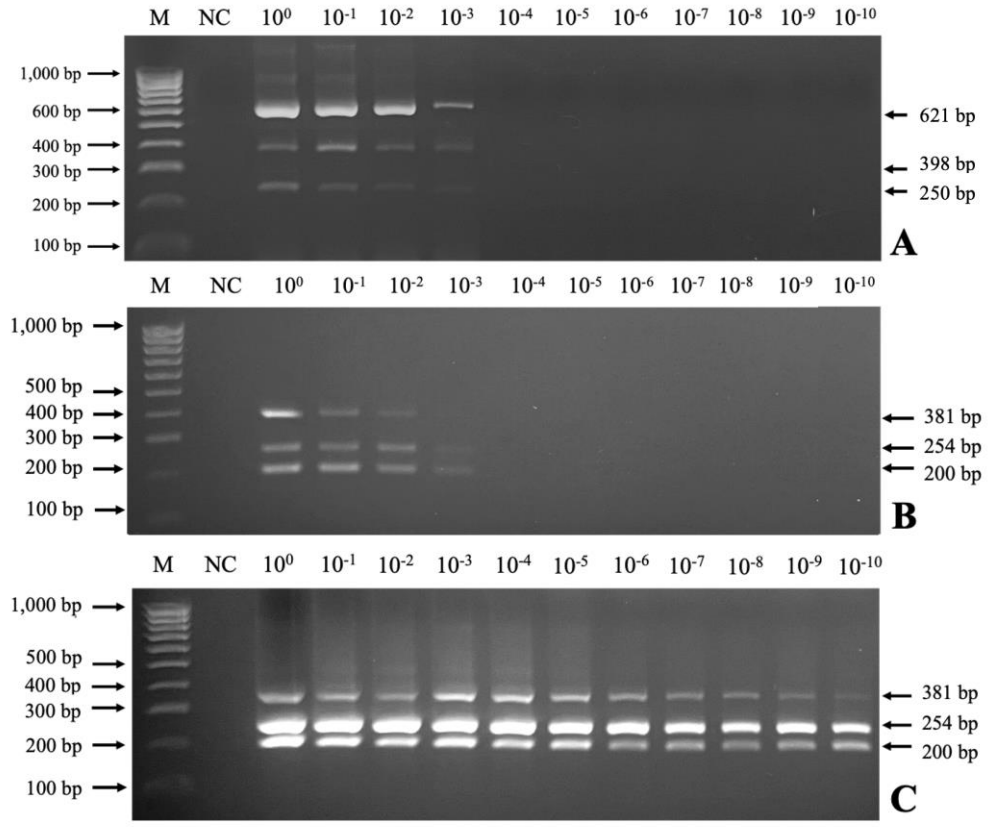

Figure 2. Sensitivity test of multiplex RT-PCR and multiplex nested RT-PCR assays by using ten-fold serial dilution of cDNA and first round PCR products. M: 100 bp + 1.5 Kb DNA Ladder (SibEnzyme, Russia) and NC: negative control. (A) multiplex RT-PCR detection using outer primers had the limit of detection (LOD) at 10^{-1}, (B) multiplex RT-PCR detection using inner primers (nested primers) had LOD at 10^{-2} and (C) ten-fold serial dilution of the PCR product from first round PCR for multiplex nested RT-PCR detection had LOD at 10^{-9}

Specificity test of multiplex RT-PCR and multiplex nested RT-PCR

The specificity assays in multiplex RT-PCR and multiplex nested RT-PCR revealed that both techniques did not cause the cross reactivity with other viruses from different genera and a healthy plant sample. Only PCR products that specific to CChMVd, CSVd and CVB were observed from both multiplex and multiplex nested RT-PCR (Figure 3A and B). The results showed that

multiplex PCR-based techniques improved in this study had the specificity to target pathogens without cross reactivity.

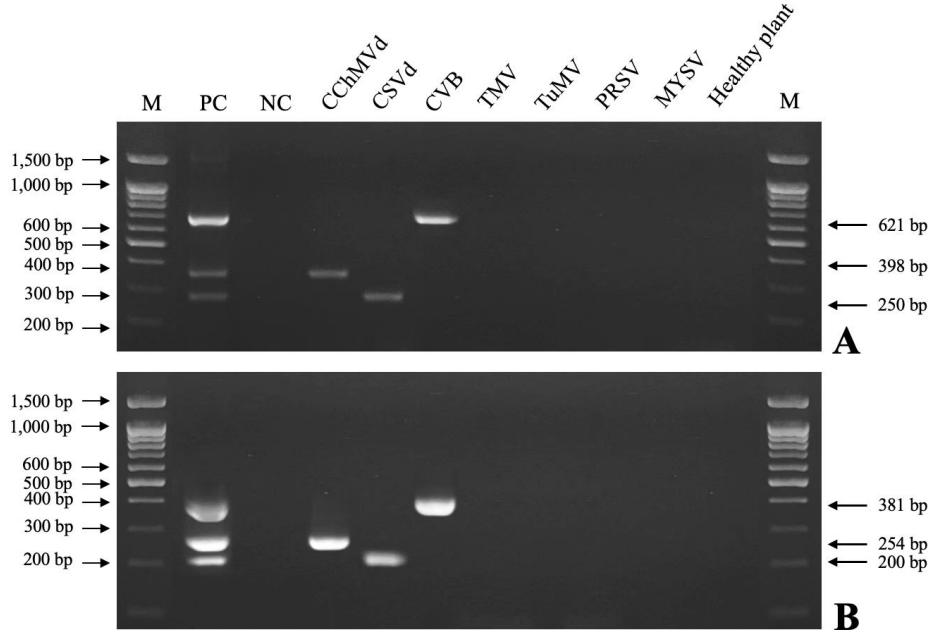

Figure 3. Specificity test of multiplex RT-PCR and multiplex nested RT-PCR. M: 100 bp + 1.5 Kb DNA Ladder (SibEnzyme, Russia), PC: positive control and NC: negative control. (A) Multiplex RT-PCR detection and (B) multiplex nested RT-PCR showed three bands of PCR products specific to CChMVd, CSVd and CVB without cross reactivity to other viruses and a healthy plant sample

Application of multiplex nested RT-PCR in actual samples

Multiplex RT-PCR detection of the 16 randomly selected samples revealed that nine out of 15 samples were positive to single infection of CVB, CChMVd, and CSVd for three, two, and two samples, respectively (Figure 4A). One sample showed co-infections of CVB and CChMVd (Figure 4A lane 5), and multiple infections of all three pathogens were found from two samples (Figure 4A lane 3 and 14). We also detected the 398 bp amplicon of CChMVd in lane 9 sample, but this showed slightly fade amplicons.

Application of multiplex nested RT-PCR, by using PCR products from the first round PCR as templates, found that all positive samples from the first round PCR were still detected the shorter fragments of CChMVd (254 bp), CSVd (200 bp), and CVB (381 bp) (Figure 4B). The sample in lane 9 that

showed fade amplicon from the first round PCR had more intensity of the amplicons after nested PCR amplification. Moreover, three samples showed negative results from the first round PCR, which detected the viroid-specific fragments in the second-round PCR, including lane 2 for CSVd and lane 6 and 8 for CChMVd (Figure 4A and B lane 2, 6 and 8).

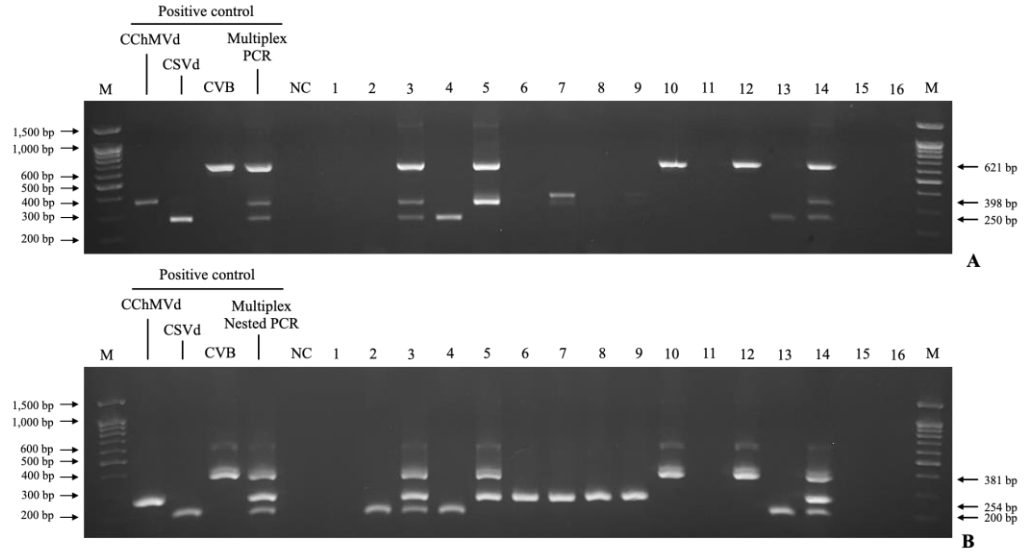

Figure 4. Detection of mixed infections of CVB, CChMVd and CSVd in chrysanthemum by using multiplex RT-PCR and multiplex nested RT-PCR. M: 100 bp + 1.5 Kb DNA Ladder (SibEnzyme, Russia), NC: negative control using DEPC water. (A) multiplex RT-PCR detection, CChMVd: 398 bp, CSVd: 250 bp, CVB: 621 bp, lane 1: healthy plant, lane 2-16: randomly selected samples. (B) multiplex nested RT-PCR, CChMVd: 254 bp, CSVd: 200 bp: CVB: 381 bp, lane 1: healthy plant, lane 2-16: randomly selected samples

Sequence analysis

Sequence analysis showed that all amplicons obtained from the second round PCR were 100% identical compared to amplicons obtained from the first round PCR of all pathogens (data not shown). Sequences of the first-round PCR of CChMVd (398 bp), CSVd (354 bp), and CVB (621 bp) were compared to corresponding sequences in GenBank using BLASTN analysis and found that five sequences of CVB shared 97.89–98.62% identity and accession numbers were MZ328198, MZ328199, MZ328200, MZ328201 and MZ328202. The whole-genome of seven sequences of CChMVd shared 93.15–97.55% identity and accession numbers were MZ328179, MZ328180, MZ328181, MZ328182,

MZ328183, MZ328184 and MZ328185. Five whole-genome sequences of CSVd shared 95.44–98.29% identity and accession numbers were MZ328188, MZ328189, MZ328190, MZ328191 and MZ328192.

Discussion

In Thailand, only CSVd has been reported to infect chrysanthemum (Netwong *et al*., 2020). However, in this study, we detected other viral pathogens from chrysanthemum that were CVB and CChMVd for the first time. Moreover, multiple infections of these three pathogens were also detected by using multiplex RT-PCR and multiplex nested RT-PCR. However, to confirm their pathogenicity as a pathogen of chrysanthemum, mechanical inoculation is needed for the futher study.

Multiplex RT-PCR has the potential to reduce the cost of diagnosis by reducing the number of assays to be performed in the detection of several viruses and viroids in a sample (Hosokawa *et al*., 2007). Song *et al*. (2012) successfully developed multiplex RT-PCR for simultaneously detecting TAV, CVB, CChMVd, and CSVd, and used this approach for routine diagnosis of virus and viroid diseases in chrysanthemum. Next, Zhao *et al*. (2015) developed multiplex RT-PCR for simultaneous detection of five viruses and two viroids consisting of TAV, CVB, CMV, TMV, PVY, CChMVd, and CSVd infecting chrysanthemum. This concluded that multiplex RT-PCR was useful for routine diagnosis of virus and viroid diseases in chrysanthemum. In this study, the primer pairs from different publications were selected to combine and perform multiplex RT-PCR and multiplex nested RT-PCR assays. The improved multiplex RT-PCR and multiplex nested RT-PCR represented high sensitivity of the detection along with the specificity to target pathogens without cross reactivity.

Plant materials often contain inhibitors that reduce the yield and quality of the RT-PCR amplification. Concomitantly, pathogens, especially virus and viroid, are often in low titer in hosts. The increase in sensitivity by reamplification of the targets is frequently required (nested or hemi-nested PCR assays) from the products obtained in the first round of amplification (Bertolini *et al*., 2002). Furthermore, nested RT-PCR assay was commonly used for viroid detection (Chung *et al*., 2005; Guan *et al*., 2017; Ebata *et al*., 2019).

The study shows that after a great number of PCR cycles (40 cycles), this ended up with smear from the products and with little satisfaction from viroids detection. Using nested PCR increased the intensity of PCR products and detected the products that did not detect or showed faint or slight appearance by the first-round PCR. Therefore, the combined detection of RT-PCR and nested

PCR was suggested for the successful diagnosis of viroids. Application of the multiplex nested PCR can greatly improve the sensitivity of the detection but also reduce cost, time, and handling (Jinxia *et al*., 2011). Recently, real-time PCR (qPCR) for quantitative detection with high sensitivity has been used for virus and viroid detection (Seigner *et al*., 2020), but qPCR machines may be expensive. Furthermore, the detection of target virus and viroid, but RT-PCR or nested PCR, is sufficient if quantification is not needed (Song *et al*., 2012).

In this study, CVB, CChMVd, and CSVd were successfully detected using three specific outer and three specific inner primer pairs in a single RT-PCR and nested PCR, respectively. In contrast, no products were detected from the negative control. When combinations of outer and inner primer pairs were used, multiplex PCR and multiplex nested PCR achieved to detect multiple infections of virus and viroid infecting chrysanthemum in Thailand for the first time. Moreover, nested PCR could amplify amplicons specific to viroids that were not detected by RT-PCR. This summarized that multiplex nested RT-PCR is a useful technique to detect multiple infections of virus and viroid in chrysanthemum based on its sensitivity, specificity, rapidness, and simultaneous detection efficacies.

Acknowledgements

This research work was supported by Royal Project Foundation (Grant No. 3060-A149), Thailand Graduate Institute of Science and Technology (TGIST) (Grant No. SCA-C0-2562-9705-TH).

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(Received: 12 August 2021, accepted: 30 January 2022)