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Development of real-time polymerase chain reaction (qPCR) technique for quantitative detection of chrysanthemum chlorotic mottle viroid (CChMVd) and chrysanthemum stunt viroid (CSVd) in chrysanthemum

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Abstract Chrysanthemum is a famous ornamental plant in the world, especially in Thailand. The production and propagation of chrysanthemum are achieved by vegetative propagation, which is a fast, easy, convenient method and many plantlets are obtained. However, this method is risk for viroid contamination. To disinfect viroid contamination, the meristem tip culture has been used for generating viroid-free plantlets. The real-time polymerase chain reaction (qPCR) which was developed to detect chrysanthemum chlorotic mottle viroid (CChMVd) and chrysanthemum stunt viroid (CSVd) from chrysanthemum plantlets, obtained from meristem tip culture for verification of viroid-free plantlets production, using new sets of primers showed that annealing at 50 ℃ was suited for both CChMVd and CSVd detection. The standard curve analysis was illustrated and found that the lowest quantification cycle (Cq) values for detecting CChMVd and CSVd were 5.42 and 18.81, respectively. Moreover, the lowest copies number of CChMVd and CSVd which could be detected were 1×10^8 and 1×10^9 copies/µl, respectively. The qPCR techniques were applied to detect both viroids from chrysanthemum plantlets and revealed that all plantlets were free from viroid-contamination. Therefore, the qPCR with high sensitivity and specificity can be used as a main technique for detecting CChMVd and CSVd in chrysanthemum.

Keywords: Quantitative PCR, Molecular detection, Viroid, Meristem tip culture

Introduction

Chrysanthemum (*Chrysanthemum x morifolium*) is one of the most important ornamental plants in the world with a high ornamental value, occupying an irreplaceable position in international flower industry (Fan *et al*.,

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2015). Many chrysanthemum cultivars are susceptible to viroid infection and there were 2 viroids which infect chrysanthemum consisting of chrysanthemum chlorotic mottle viroid (CChMVd) and chrysanthemum stunt viroid (CSVd) (Cho *et al*., 2013).

Viroids are small (250–400 nt), circular, highly structured RNAs that do not encode proteins and they are replicated by nuclear or chloroplastic DNA-dependent RNA polymerases that accept RNA templates of the host plants (Ding and Itaya, 2007). CChMVd belongs to the genus *Pelamoviroid* of the family *Avsunviroidae* (de la Pena *et al*., 1999) and CSVd belongs to the genus *Pospiviroid* of the family *Pospiviroidae* (Diener and Lawson, 1973). CChMVd infects only chrysanthemum while CSVd infects several host plants such as *Petunia x hybrida*, tomato, *Gynura aurantiaca*, *Ageratum* sp., *Dahlia* sp., *Argyranthemum frutescens* and many plants belonging to the families Solanaceae and Asteraceae (Cho *et al*., 2013; Matsushita *et al*., 2019; Gobatto *et al*., 2019).

CChMVd usually induced mottling symptoms and some tolorent cultivars showed symptomless (Yamamoto and Sano, 2005). CSVd induced several symptoms, and the most unique symptoms were stunting and other symptoms such as yellow spots, reduction in the size of leaf, flower, and poor rooting ability (Matsushita, 2013).

Viroids can contaminate in chrysanthemums, especially in main commercial cultivars. Traditionally, chrysanthemum is propagated by using a vegetative propagation to obtain many numbers of shoots, but this method was risk to obtain viroid-contaminated shoots if the mother stock plant was infected (Cho *et al*., 2013). Generally, to produce viroid-free chrysanthemum plantlets, a meristem tip culture technique is used (Verma *et al*., 2004). The verification of viroid-free chrysanthemum plantlets after meristem tip culture is neccessary, the sensitive and accuracy detection technique is strongly required (Supakitthanakorn *et al*., 2022a).

To detect plant viruses and viroids, PCR-based techniques were commonly used (Webster *et al*., 2004). The qPCR has been recently used with its efficiency to detect quantitatively in real-time and no need of gel electrophoresis for results analysis leading to reduction cost and time of the detection (Lopez *et al*., 2008). The work aimed to develop the qPCR technique for detecting CChMVd and CSVd from chrysanthemum plantlets obtained from meristem tip culture for verifying viroid-free plantlet production.

Materials and methods

Construction of viroid recombinant plasmids

CCMVd HL4 variant (GenBank accession no. MZ328193.1) and CSVd CM4 variant (GenBank accession no. MZ328193.1) which were detected from a previous study (Supakitthanakorn *et al*., 2022b) was used as DNA templates for developing the qPCR technique. The PCR product of whole genome of both viroids were amplified by using viroid specific primers (Table 1). The PCR amplifications of full-length sequences were performed by using EconoTaq® PLUS & PLUS GREEN 2X Master Mixes (Lucigen, USA) in the GeneMax PCR thermal cycler (Bioer, China). PCR mixture contained 12.5 µl of 2X PCR master mix, 1.0 µl (10 µM) of each forward and reverse primers, 1.0 µl of cDNA and 9.5 µl of DEPC-treated water for final volume of 25 µl. PCR cycles were started at 94 ℃ for 5 min, 40 cycles of 94 ℃ for 30 sec, 56 – 59 ℃ (Table 1) for 30 sec, 72 ℃ for 45 sec and 72 ℃ for 7 min. PCR products were visualized on 2.0% agarose gel electrophoresis stained by RedSafe™ (iNtRON, Korea). Subsequently, the PCR product was purified by using PCR Clean Up & Gel Extraction Kit (Bio-Helix, Taiwan) and then the purified PCR products were cloned into pCR™ 2.1-TOPO® vector (Invitrogen, USA). The recombinant plasmid was transformed into TOP10™ chemically competent *Escherichia coli* cells (Invitrogen, USA). The plasmids were extracted by using Plasmid Mini Prep (Bio-Helix, Taiwan) and verified by sequencing. Then, the plasmid was used as a template for further experiments.

Primer design and optimization

qPCR primers were designed using sequences of CChMVd HL4 (GenBank accession no. MZ328182.1) and CSVd CM4 (GenBank accession no. MZ328193.1) as templates which were performed in Custom Oligos & qPCR Probes Online Tools (Sigma Aldrich, USA) (http://www.oligoarchitect.com/ LoginServlet) with default setting.

The qPCR cocktail contained 10.0 µl of THUNDERBIRD™ SYBR™ qPCR Mix (Toyobo, Japan), 0.5 µl of 10 µM (final concentration 0.5 µM) of each forward and reverse primer, 1.0 µl of a recombinant plasmid (1.0 µg/µl) and 8.0 µl of nuclease-free water to adjust a final volume of 20.0 µl. qPCR reactions were performed in CFX96 1000c Touch Real-Time PCR Detection System (Bio-Rad, USA) and the conditions were done as follows; denaturation at 95 ℃ for 1 min, 40 cycles of denaturation at 95 ℃ for 15 sec, annealing at 50/55/60 ℃ for 30 sec. The melting curve analysis was performed at 95 ℃ for 10 sec, 65 ℃ for 5 sec, then followed by a slow increase from 65 ℃ to 95 ℃ with a speed of 0.5 ℃ per sec. The experiment was repeated triplicates. The melt curve is used to view the real-time data for each fluorophore relative fluorescent unit (RFU) per temperature for each well. The melt peak analysis is

used to view the negative regression of the RFU data per temperature for each well.

Specificity assay

To determine the specificity of the assay, cDNA of other chrysanthemum-infecting viruses including cucumber mosaic virus (CMV), chrysanthemum virus B (CVB), tobacco mosaic virus (TMV) and turnip mosaic virus (TuMV) obtained from the previous study (Supakitthanakorn *et al*., 2022c). The reverse transcription (RT)-qPCR cocktail and reaction were set up as described above. CChMVd and CSVd plasmids were used as the positive control.

Standard curve analysis

Seven-fold serial dilution (10^{0} – 10^{-7}) of recombinant plasmids of both viroids was prepared by decanting 5.0 µl of original recombinant plasmid to other tubes containing 45.0 µl of nuclease-free water. Measurement of diluted plasmid DNA quantity by using NanoDrop 2000c System (Thermo Fisher Scientific, USA). The qPCR reaction was set up as described previously.

Evaluation of RT-qPCR for detecting viroids from chrysanthemum plantlets

To estimate the efficiency of developed qPCR protocols for detecting CChMVd and CSVd from chrysanthemum plantlets obtained from meristem tip culture were used to verify viroid-free plantlet production. Total RNA was extracted from upper leaves by using NucleoSpin™ RNA Plant and Fungi (Macherey-Nagel, Germany) according to the manufacturer's instructions and the cDNA was synthesized using ReverTra Ace™ qPCR Master Mix with gDNA Remover (Toyobo, Japan) according to the manufacturer's instruction. The RT-qPCR reaction was set as previously described previously.

Results

Primer design and optimization

qPCR primers were designed, and primer sequences were shown in Table 1. To determine the optimal annealing temperature of qPCR reactions, the annealing temperatures were conducted at 50, 55 and 60 ℃ for both CChMVd and CSVd. The results showed that cycle quantification (Cq) values of CChMVd were 5.42, 5.76 and 7.24 obatined from annealing at 50, 55 and 60 ℃, respectively, and the T_m was 83.00 ℃ (Figure 1A – C). The Cq values of CSVd

were 18.81, 19.66 and 25.14 obatined from annealing at 50, 55 and 60 ℃, respectively, and the T_m was 82.00 ℃ (Figure 1D – F).

The melt peak analysis showed that all tested peak lines were higher than the threshold line (the straight green line) which mean all tested peak lines were considered to positive (Figure 1C and F). While the non-template control (NTC) using nuclease-free water instead of DNA had Cq value for 39.85 (Figure 1A), but the melt peak analysis revealed that the T_m of NTC could not be calculated (N/A) which mean NTC was negative (Figure 1C and F). Therefore, the result showed that even the Cq values of the sample were showed, but it did not mean that the sample was positive. The melt peak analysis must be determined along with the quantification plot to confirm the results.

Table 1. PCR and qPCR primers for detection of CChMVd and CSVd used in this study

Technique	Viroid	Primer name	Sequence (5'-3')	T_m* (℃)	Product size (bp)	Reference
PCR	CChMVd	CCh-nF	CAGGATCGAAACCTCTTCCA	59	398	Ebata *et al.* (2019)
		CCh-MR1	GACCTCTTGGGGGTTAGAAA			
PCR	CSVd	CSVd-F	TTCTTTCAAAGCAGCAGGGT	56	354	Chung *et al.* (2006)
		CSVd-R	AAAGAAATGAGGCGAAGAAG			
qPCR	CChMVd	qCCh21F	TGATGAAGATCCATGACAG	59	150	This study
		qCCh171R	TTACCTCTACTCCGCTTA			
qPCR	CSVd	qCS148F	TGCGAGACAGGAGTAATC	60	204	This study
		qCS354R	AGGGAACAAAACTAAGGTT			

*T_m = melting temperature

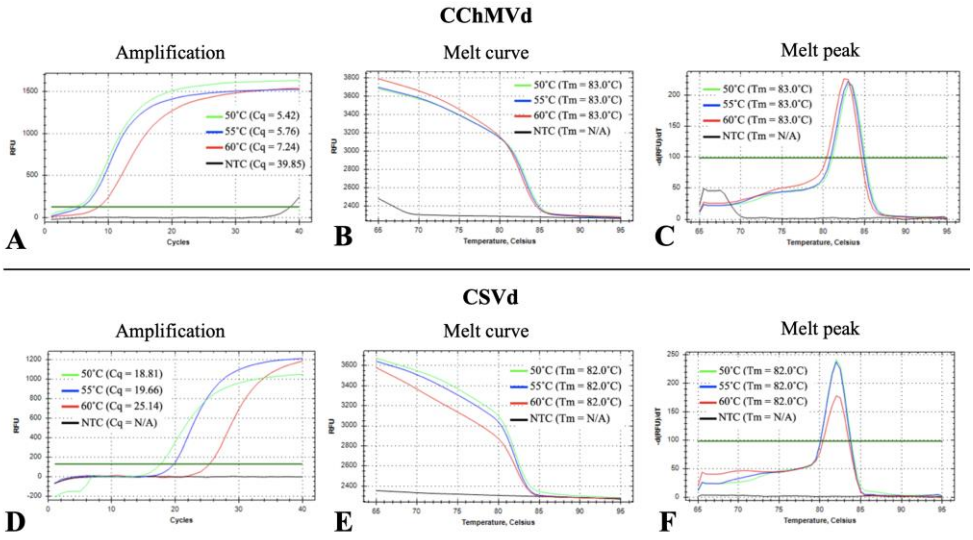

Figure 1. Optimization of qPCR reaction for CChMVd (A – C) and CSVd (D – E). (A and D) annealing temperature validating at 50, 55 and 60 ℃, (B and E) melt curve analysis and (C and F) melt peak analysis

Specificity assay

To determine the possibility of cross reaction, RT-qPCR was performed according to the optimal conditions described previously compared to cDNA of other positive viruses and a viroid. The results of specificity assays showed that the Cq value and melt peak analysis were observed from PC (positive control using recombinant plasmids of CChMVd and CSVd) while other tested viruses and non-template control were not detected (Figure 2). This concluded that RT-qPCR was specific to only individual CChMVd and CSVd.

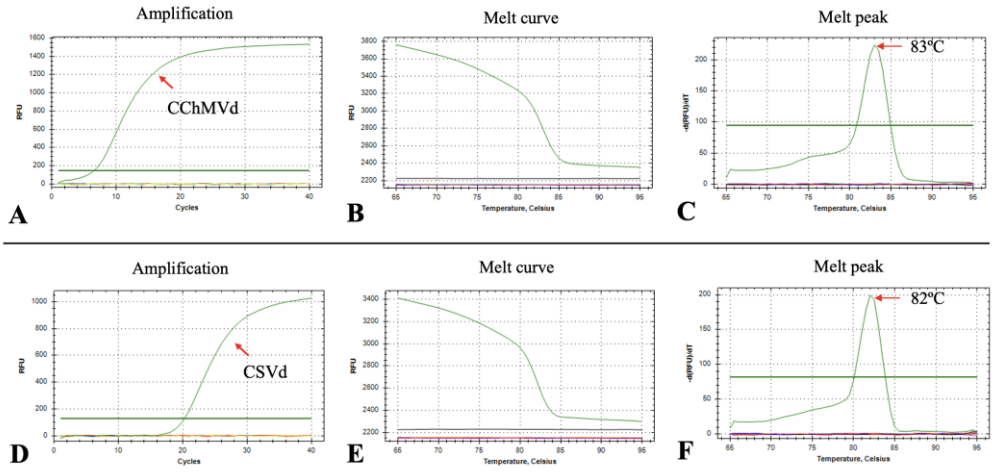

Figure 2. Specificity assays of qPCR for detection of CChMVd (A – C) and CSVd (D – F) using recombinant plasmid DNA as templates. (A) amplification plot at Cq = 8.54 of CChMVd, (B) melt curve analysis, (C) melt peak analysis at T_m = 83.00 ℃ of CChMVd, (D) amplification plot at Cq = 20.12 of CSVd, (E) melt curve analysis and (F) melt peak analysis at T_m = 82.00 ℃ of CSVd. Other tested viruses consisting of CMV, CVB, TMV and TuMV were negative (below the threshold line)

Standard curve analysis

The standard curve was conducted by using recombinant plasmids of CChMVd and CSVd which were 7–fold diluted (10^0 – 10^{-7}). The results showed that the concentrations of recombinant plasmids of CChMVd and CSVd were were 1 µg/µl, 100 ng/µl, 10 ng/µl, 1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl and 100 fg/µl measured by using a NanoDrop 2000c (Thermo Fisher, USA). The Cq value, copy nunber and T_m of CChMVd were shown in Table 2 and Figure 3 and the data of CSVd were shown in Table 3 and Figure 4. The

standard curves were used as a standard reference for the extrapolation of quantitative information for CChMVd and CSVd targets of unknown concentrations in chrysanthemum plants.

Table 2. Standard curve analysis of CChMVd

Concentration	Cq* (± SD)	Copy number	T_m (℃)	Result
Standard 1 (1 µg/µl)	8.56 ± 0.05	1×10^{15} copies/µl	83.0	Positive
Standard 2 (100 ng/µl)	11.91 ± 0.13	1×10^{14} copies/µl	83.0	Positive
Standard 3 (10 ng/µl)	16.05 ± 0.09	1×10^{13} copies/µl	83.0	Positive
Standard 4 (1 ng/µl)	20.00 ± 0.07	1×10^{12} copies/µl	83.0	Positive
Standard 5 (100 pg/µl)	23.44 ± 0.13	1×10^{11} copies/µl	83.0	Positive
Standard 6 (10 pg/µl)	27.23 ± 0.05	1×10^{10} copies/µl	83.0	Positive
Standard 7 (1 pg/µl)	31.41 ± 0.12	1×10^{9} copies/µl	83.0	Positive
Standard 8 (100 fg/µl)	34.92 ± 0.11	1×10^{8} copies/µl	83.0	Positive
Non-template control	N/A	N/A	N/A	Negative

*Average from 3 replicates, SD = standard deviation

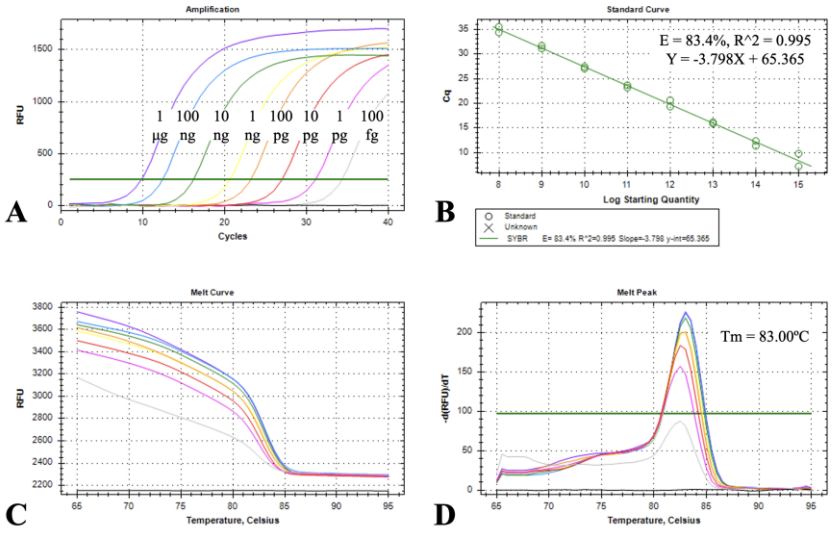

Figure 3. Standard curve analysis to measure a copy number of recombinant plasmid of CChMVd. (A) quantitative amplification, (B) standard curve analysis. The x-axis indicates the copy number, ranging from $1 \times 10^{8} - 1 \times 10^{15}$ copies/µl, used in the experiments. The y-axis represents the corresponding cycle quantification (Cq) values, (C) melt curve analysis and (D) melt peak analysis

Table 3. Standard curve analysis of CSVd

Concentration	Cq* (±SD)	Copy number	T_m (℃)	Result
Standard 1 (1 µg/µl)	19.29 ± 0.01	1×10^{15} copies/µl	82.0	Positive
Standard 2 (100 ng/µl)	22.21 ± 0.06	1×10^{14} copies/µl	82.0	Positive
Standard 3 (10 ng/µl)	25.14 ± 0.07	1×10^{13} copies/µl	82.0	Positive
Standard 4 (1 ng/µl)	27.69 ± 0.19	1×10^{12} copies/µl	82.0	Positive
Standard 5 (100 pg/µl)	31.96 ± 0.14	1×10^{11} copies/µl	82.0	Positive
Standard 6 (10 pg/µl)	35.53 ± 0.06	1×10^{10} copies/µl	82.0	Positive
Standard 7 (1 pg/µl)	37.70 ± 0.07	1×10^{9} copies/µl	82.0	Positive
Standard 8 (100 fg/µl)	N/A	N/A	N/A	Negative
Non-template control	N/A	N/A	N/A	Negative

*Average from 3 replicates, SD = standard deviation

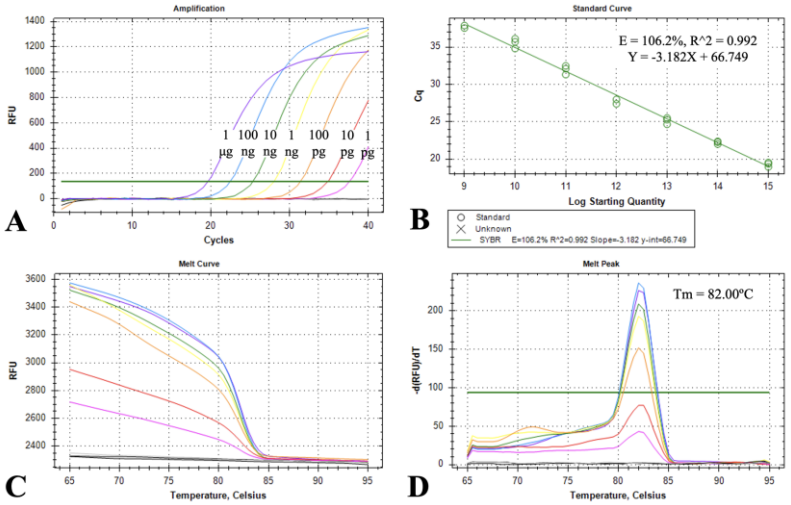

Figure 4. Standard curve analysis to measure a copy number of recombinant plasmid of CSVd. (A) quantitative amplification, (B) standard curve analysis. The x-axis indicates the copy number, ranging from $1 \times 10^{9} - 1 \times 10^{15}$ copies/µl, used in the experiments. The y-axis represents the corresponding cycle quantification (Cq) values, (C) melt curve analysis and (D) melt peak analysis

Evaluation of RT-qPCR for detecting viroids from chrysanthemum plantlets

To ensure the production of CChMVd-free chrysanthemum plantlets obtained from meristem tip culture, total of 18 chrysanthemum samples consisting of 6 cultivars consisting of White Udon, Hauy Leuk (2VF), Hauy

Leuk (3VF), White Khunwang, TW005 and Yellow Kamin (Figure 5) and each cultivar contained 3 replications were examined. Total of 18 chrysanthemum had the Cq values ranging from 30.88 – 35.54 (Figure 6A) while the recombinant plasmid of CChMVd (standard curve) had the Cq values starting at 7.50 – 38.75 (1 µg – 10 fg) (Figure 6A). Consideration of only Cq value of the amplification plot, the tested chrysanthemum samples had low titers of CChMVd. However, the melt peak and melt curve analysis confirmed that the Cq values were not from the CChMVd amplicons but were primer-dimer conformation. The T_m of plasmid DNA of CChMVd was 83.00℃ whereas the Tm of tested chrysanthemum samples were approximately 67.50℃ (Figure 6D).

For CSVd detection, total of 18 chrysanthemum plantlets had the Cq values ranging from 30.30 – 37.83 (Figure 7A) while the recombinant plasmid of CSVd had the Cq values starting at 21.10 – 37.73 (1 µg – 10 fg) (Figure 7A). Consideration of only Cq value of the amplification plot, the tested chrysanthemum samples had low titers of CSVd. However, the melt peak and melt curve analysis confirmed that the Cq values were not from the CSVd amplicons but were primer-dimer conformation. The T_m of plasmid DNA of CSVd was 82.00℃ whereas the T_m of tested chrysanthemum samples were approximately 73.50℃ (Figure 7D).

Therefore, total of 18 chrysanthemum plantlets from 6 cultivars obtained from meristem tip culture were free from CChMVd and CSVd infection which were verified by RT-qPCR detection using protocols and primers developed in this study.

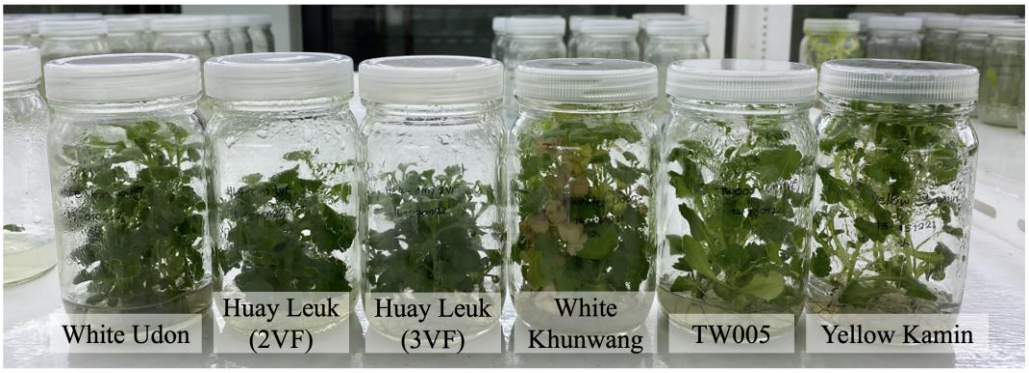

Figure 5. Chrysanthemum plantlets from 6 cultivars obtained from meristem tip culture used for detecting CChMVd and CSVd by the RT-qPCR technique

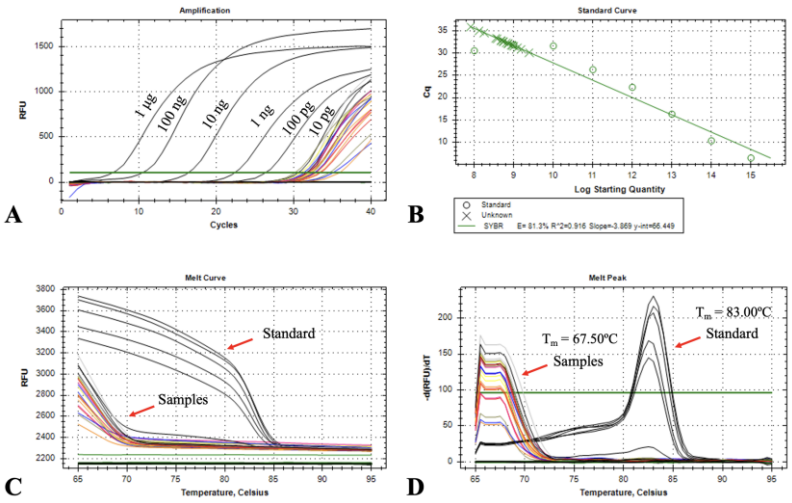

Figure 6. RT-qPCR detection of CChMVd from 18 chrysanthemum plantlets (6 cultivars and 3 replicates/each) obtained from meristem tip culture. The recombinant plasmid of CChMVd which was used as a standard was indicated by black line and the samples were indicated by multicolor lines: (A) amplification plot, (B) standard curve analysis, (C) melt curve analysis and (D) melt peak analysis. T_m of standards is 83.00 ℃ whereas T_m of samples is 67.50 ℃

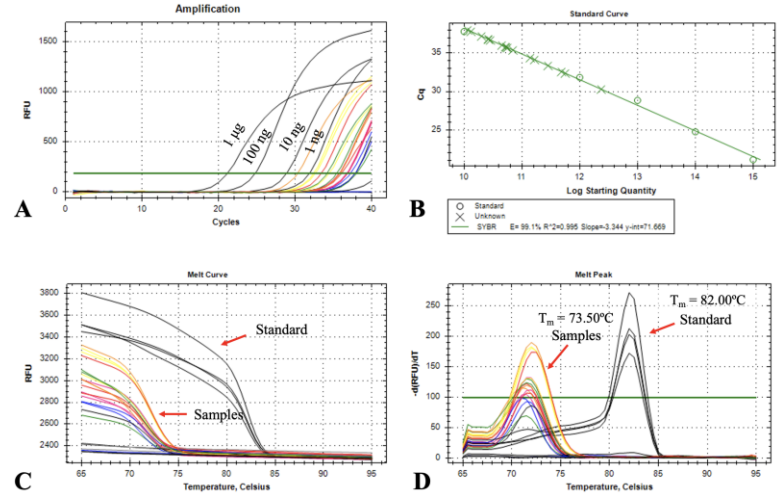

Figure 7. RT-qPCR detection of CSVd from 18 chrysanthemum plantlets (6 cultivars and 3 replicates/each) obtained from meristem tip culture. The recombinant plasmid of CSVd which was used as a standard was indicated by black line and the samples were indicated by multicolor lines: (A) amplification plot, (B) standard curve analysis, (C) melt curve analysis and (D) melt peak analysis. T_m of standards is 82.00 ℃ whereas T_m of samples is 73.50 ℃

Discussion

The qPCR has been used to detect various plant pathogens due to its sensitivity, accuracy, the number of targets could be determined and can detect pathogens present even in low quantities (Grosdidier *et al*., 2017). The use of qPCR allows to amplify targets with a size between 100–300 bp. However, amplicons more than 500 bp can also be conducted (Wilhelm *et al*., 2006). Such amplifications are more difficult with classical PCR systems because such small fragments are less easy to visualize on an agarose gel of the kind used in classical PCR (Debode *et al*., 2017). This study designed the primers for amplifying amplicons size 150 and 204 bp of CChMVd and CSVd, respectively.

A common method for validating qPCR assays involves the construction of a standard curve, enabling the determination of the efficiency, linear dynamic range, and reproducibility of a qPCR assay. The efficiency (E) of PCR is defined as the fraction of target molecules that are copied in one PCR cycle (Lalonde *et al*., 2015). The efficiency of the assay should be 90–110%, the R^2 of the standard curve should be more than 0.980 (Svec *et al*., 2015). Standard curves are constructed using a dilution series of standard control materials, typically four to six 10-fold dilution points with at least three replicates per point and a defined number of target molecules (Bivins *et al*., 2021). In this study. the standard curve generating from recombinant plasmids of CChMVd and CSVd revealed that the newly developed primers are well suited for a sensitive qPCR detection of CChMVd and CSVd with qPCR efficiencies of 83 and 106%, respectively, and R^2 values were 0.995 for CChMVd and 0.992 for CSVd. The results indicated that even the efficiency values of qPCR were lower than the standard, but the R^2 values were as of the standard. Therefore, the qPCR developed in this study was effective in detecting CChMVd and CSVd.

Only Cq values could not be used to confirm the present of pathogens or determined as a positive reaction because the formation of primer-dimer could cause the Cq values also (Garafutdinov *et al*., 2020). The melting curve and melt peak analysis should be conducted to prove the correct detection (Lalonde *et al*., 2015). In this study, the Cq values of standards and examined samples were detected with different Cq values. When the melt curve and peak analysis were conducted, the T_m of examined samples were not same as of the standards. The melt peak T_m of CChMVd standards was 83.00 ℃ but the T_m of examined samples was 67.50 ℃. The T_m of CSVd standards was 82.00 ℃ and the T_m of examined samples was 73.50 ℃. Therefore, the examined chrysanthemum plantlets samples were negative to CChMVd and CSVd considering on the the

different T_m of melt curve and peak analysis compared to the standards even the Cq values were detected.

The meristem tip culture was routinely used to eliminate viruses and viroid contaminated in chrysanthemum mother stocks (Verma *et al*., 2004). To ensure the production of virus and viroid-free chrysanthemum plantlets, the conventional RT-PCR was routinely used. However, the titer of viroid was occasionally low in plant tissues (Hosokawa *et al*., 2004; Nabeshima *et al*., 2017). This study developed and conducted RT-qPCR for detection of CChMVd and CSVd in chrysanthemum plantlets which were obtained from meristem tip culture. The results showed that qPCR could be used to detect CChMVd and CSVd effectively from the positive control or standard using recombinant plasmids of both CChMVd and CSVd whereas all chrysanthemum plantlets were negative. The RT-qPCR for detecting CSVd was previously developed (Kim *et al*., 2015), but there is no report of developing or using qPCR for detection of CChMVd. Therefore, this study is the first development of qPCR for detecting CChMVd in chrysanthemum. In conclusions, the qPCR that was developed in this study had the potential for detecting CChMVd and CSVd in chrysanthemum due to its accuracy, sensitivity and specificity and could be used to ensure the production of viroid-free chrysanthemum plantlets.

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