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ORIGINAL ARTICLE

Development of the colorimetric loop-mediated isothermal amplification technique for rapid and sensitive detection of chrysanthemum stunt viroid in chrysanthemum

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Abstract

Chrysanthemum stunt viroid (CSVd) is a serious pathogen infecting chrysanthemum worldwide. To improve and enhance the detection procedure, a colorimetric loop-mediated isothermal amplification (LAMP) technique was developed. Six LAMP primers were newly designed and tested to determine the optimal conditions using a recombinant plasmid of CSVd as a DNA template. The optimal conditions for colorimetric LAMP were incubation at 65°C for 45 min. Under these conditions, a ladder-like pattern of LAMP products was detected along with a change of color from pink to yellow in the positive reactions. Limits of the detection (LOD) of colorimetric LAMP were up to 1 fg · μl^{-1} of plasmid DNA concentration which was 10^4 times greater than that of polymerase chain reaction (PCR). The developed colorimetric LAMP was not cross reacted to other viruses and viroids. From detection of actual samples and chrysanthemum plantlets which were obtained from meristem tip culture, the colorimetric LAMP showed effective potential in detecting CSVd. Therefore, the colorimetric LAMP can be used as a main technique to detect CSVd and ensure CSVd-free chrysanthemum plantlet production due to its accuracy, rapidness and sensitivity.

Keywords: chrysanthemum stunt viroid, LAMP, molecular detection, ornamental plant, *Pospiviroid*

Introduction

Chrysanthemum (*Chrysanthemum x morifolium*) is an important and highly economic cut flower in the world due to its cultural importance and its various shapes and colors (Zalewska *et al.* 2007). Chrysanthemum propagation is achieved by vegetative propagation which is a convenient and quick method and can obtain a large number of shoots (Verma *et al.* 2004). However, this method carries a high risk from an infected mother plant, especially viral and viroid diseases (Cho *et al.* 2013). Strategies for management of viroid diseases in chrysanthemum include establishment of viroid-free plantlets (Hosokawa *et al.* 2004).

Viroids are the smallest plant pathogens (250–400 nucleotides). They are circular, highly-structured RNAs that do not encode proteins and are replicated by nuclear or chloroplastic DNA-dependent RNA polymerase forced to accept viroid RNA templates of the host plants (Ding and Itaya 2007). Chrysanthemum plants are infected by two viroids, chrysanthemum chlorotic mottle viroid (CChMVd) and chrysanthemum stunt

viroid (CSVd). However, CSVd seems to cause more serious damage than CChMVd (Cho *et al*. 2013).

CSVd belongs to the genus *Pospiviroid* of the family Pospiviridae which is approximately 354 nucleotides long (Haseloff and Symons 1981; Doi and Kato 2004). Members of the Pospiviroidae family have a rod-like structure including a central conserved region (CCR). They replicate in the nucleus of infected cells, and lack ribozyme activity (Di Serio and Flores 2008). CSVd was first discovered from chrysanthemums which showed stunting in the United States and was subsequently found in chrysanthemum-growing countries (Horst *et al*. 1977). CSVd infection induces symptoms such as stunting, yellow spots, reduction of leaf and flower size, and poor rooting ability (Matsushita 2013). However, the symptoms induced by CSVd depend on the cultivar and the environment (Horst *et al*. 1977). Natural hosts of CSVd include chrysanthemum, petunia (*Petunia x hybrida*), potato and *Oxalis latifolia* (Matsushita *et al*. 2019; Gobatto *et al*. 2019).

To detect viroids, reverse transcription polymerase chain reaction (RT-PCR) was commonly used. However, PCR requires a thermal cycler and a gel electrophoresis system which involve expensive and lengthy procedures (Jeong *et al*. 2014). Therefore, the loop-mediated isothermal amplification (LAMP) was developed. LAMP was first developed by Notomi *et al*. (2000). LAMP amplification is achieved by the strand displacement ability of *Bst* polymerase which is active under isothermal conditions. LAMP is a well-established technique and applied in many fields, such as medicine, agriculture, and food industries due to its simplicity, specificity, rapidity, and low-cost (Panno *et al*. 2020). Traditionally, LAMP is used for DNA amplification, but for amplifying RNA, such as viruses and viroids, the reverse transcription (RT)-LAMP has been applied (Fukuta *et al*. 2004). Moreover, to reduce the risk of contamination and enhance the rapidness, one step RT-LAMP was developed to detect pepino mosaic virus (PepMV) from tomato (Hasiów-Jaroszewska and Borodynko 2013). Recently, LAMP has been widely used to detect plant pathogens, especially viruses and viroids (Panno *et al*. 2020), such as, tobacco mosaic virus (TMV) (Supakitthanakorn *et al*. 2022b), maize chlorotic mottle virus (MCMV) (Liu *et al*. 2016), mirafiori lettuce big vein virus (MiLBVV) (Almasi 2017), columnea latent viroid (CLVd) (Bhuvitarkorn *et al*. 2019) and plum viroid I (PVdI) (Bester and Maree 2022). To avoid post-amplification contamination, the colorimetric LAMP has been applied to detect viruses and viroids (Bhuvitarkorn *et al*. 2019; Supakitthanakorn *et al*. 2022b). Positive and negative colorimetric detection results are easily distinguishable by a visible color change, which reduces the complexity and duration of the detection procedure (Poole *et al*. 2017). Therefore, this study was aimed to develop the recent colorimetric LAMP for enhancing the effectiveness, rapidness and convenience of CSVd detection from chrysanthemum.

Materials and Methods

Construction of CSVd recombinant plasmid

CSVd CM4 variant (GenBank accession no. MZ328193.1), detected from a previous study (Supakitthanakorn *et al*. 2022a), was used as a template for developing the colorimetric LAMP technique. The PCR product of whole genome of CSVd was amplified by using CSVd-specific primers consisting of CSVd-F (5'-TTCTTTCAAAGCAGCAGGGT-3') and CSVd-R (5'-AAAGAAATGAGGCGAAGAAG-3') (Chung *et al*. 2006). The PCR amplification of a full-length sequence (354 bp) of CSVd was performed by using EconoTaq® PLUS & PLUS GREEN 2X Master Mixes (Lucigen, Wisconsin, USA) in a GeneMax PCR thermal cycler (Bioer, Zhejiang, China). The 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 a final volume of 25 µl. PCR cycles were started at 94°C for 5 min, 40 cycles at 94°C for 30 s, 54–59°C (Table 1) for 30 s, 72°C for 45 s and 72°C for 7 min. PCR products were visualized on 2.0% agarose gel electrophoresis stained by RedSafe™ (iNtRON, Gyeonggi, Korea). Subsequently, the PCR product was purified by using PCR Clean Up & Gel Extraction Kit (Bio-Helix, City, Taiwan) and then the purified PCR products were cloned into pCR™ 2.1-TOPO® vector (Invitrogen, Waltham, USA). The recombinant plasmid was transformed into TOP10™ chemically competent *Escherichia coli* cells

Table 1. LAMP primers that were designed and used in this study for detecting CSVd

Primer name	Sequence (5′-3′)	Position (5′-3′)
FIP	TTCCCCGGGGATCCCTGAAG-	F2: 34–53
	TGACCCTGCTGCTTTGAAAG	F1c: 78–97
BIP	GGAAGTCCGACGAGATCGCG-	B2: 165–184
	GGGTGAAAACCCTGTCTAGG	B1c: 105–124
F3	TTGTGGTTCCTGTGGTGC	11–28
B3	TCCAGGAGAGGAAGGAAACT	192–211
LF	GACTTCTTCGCCCTATTTCTTT	56–77
LB	CACTCCTGCGAGACAGGAGTAA	142–163

FIP – forward inner primer (consisting of F2 and F1c primers), BIP – backward inner primer (consisting of B2 and B1c primers), F3 – forward primer, B3 – backward primer, LF – loop-forward primer, LB – loop backward primer

(Invitrogen, Waltham, USA). The recombinant plasmids were extracted by using Plasmid Mini Prep (Bio-Helix, City, Taiwan) and verified by sequencing. Then, the recombinant plasmid was used as a template for further experiments.

LAMP primer design

Six LAMP primers consisting of forward inner primer (FIP), backward inner primer (BIP), forward primer (F3), backward primer (B3), loop-forward (LF) and loop-backward (LB) were designed based on the whole genome sequence of CSVd (GenBank accession no. AB255880.1) retrieved from GenBank which was performed by using NEB® LAMP Primer Design Tool (https://lamp.neb.com) (New England Biolabs, Ipswich, USA) with default setting. All LAMP primers verified the specificity of CSVd by blast and multiple alignment analysis.

Optimization of LAMP reaction

Colorimetric LAMP reactions were performed by using WarmStart® Colorimetric LAMP 2X Master Mix with UDG (New England Biolabs, Ipswich, USA). A total of 25.0 µl of LAMP component contained 12.5 µl of WarmStart® Colorimetric LAMP 2X Master Mix with UDG, 2.5 µl of LAMP primer mix (10X), 1.0 µl of plasmid DNA (1 $\mu g \cdot \mu l^{-1}$) and 9.0 µl of nuclease-free H_2O. The LAMP reactions were initially tested in a dry bath incubator by incubation at 61, 63, 65 and 67°C for 60 min each. To determine which temperature successfully amplified LAMP products the time of incubation was varied for 30, 45 and 60 min.

The LAMP products were analyzed on 1.2% agarose gel electrophoresis and the gel was stained by RedSafe™ Nucleic Acid Staining Solution (iNtRON, Gyeonggi, Korea). To determine the colorimetric results, the color of LAMP reaction that changed from pink to yellow after incubation was considered as a positive result whereas the negative result remained pink.

Sensitivity assay

To evaluate limits of the detection (LOD) of LAMP, the CSVd plasmid was prepared by using a ten-fold serial dilution method and the amount of plasmid was measured by using NanoDrop 2000c (Thermo Fisher, Waltham, USA). The LAMP reaction was performed using the optimal conditions for LAMP detection and LAMP products were visualized according to a previously described method. The PCR was used to compare the LOD of LAMP technique. The PCR amplification was conducted as previously described and the recombinant plasmid was used as a template.

Specificity assay

To determine possible cross reactivity to other viruses and viroids, the positive cDNA of other viruses and viroids including cucumber mosaic virus (CMV), chrysanthemum virus B (CVB), tobacco mosaic virus (TMV), turnip mosaic virus (TuMV) and chrysanthemum chlorotic mottle viroid (CChMVd) were examined. CMV was isolated from tobacco while CVB, TMV, TuMV and CChMVd were isolated from chrysanthemum (Supakitthanakorn *et al.* 2022a; Supakitthanakorn *et al.* 2022b). Total RNA of all viruses and CChMVd were isolated from infected leaves by using TRIzol® Reagent (Invitrogen, Waltham, USA) according to the manufacturer's instructions and then used as templates for cDNA synthesis by using ReverTra Ace™ qPCR Master Mix with gDNA Remover (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Briefly, 2.0 µl of total RNA was added to the RT mixture containing 2.0 µl of 4 DN Master Mix, 4.0 µl of nuclease-free water and incubated at 37°C for 5 min. After incubation, 2.0 µl of 5x RT Master Mix II was added and subsequently incubated at 37°C for 15 min, 50°C for 5 min and 98°C for 5 min. The cDNA was stored at –20°C before use. The PCR primers used for detection of those viruses and CChMVd are shown in Table S1. The LAMP reaction was performed using the optimal conditions for LAMP detection and LAMP products were visualized using a previously described method.

Evaluation of colorimetric LAMP in detection of CSVd from actual samples

To evaluate the detection efficiency of the developed colorimetric LAMP technique, the CSVd-positive cDNA which were synthesized previously from chrysanthemum samples were examined along with the newly extracted RNA from 12 chrysanthemum plantlets (1 month old) obtained from meristem tip culture. Total RNA was extracted from shoots by using NucleoSpin™ RNA Plant and Fungi (Macherey-Nagel, Nordrhein-Westfalen, Germany) according to the manufacturer's instructions and the cDNA was synthesized by using ReverTra Ace™ qPCR Master Mix with gDNA Remover (Toyobo, Osaka, Japan) according to the manufacturer's instructions as described previously. The LAMP reaction for detecting CSVd was performed to determine the optimal conditions. To compare the detection efficiency of LAMP, the PCR was conducted. The PCP reaction was performed as previously described.

Results

LAMP primers design

Six LAMP primers, including FIP, BIP, F3, B3, LF and LB, were designed and their positions on the genome of CSVd are shown in Table 1. Primer stock concentrations were prepared as follows: 16 µM of FIP and BIP, 8 µM of F3 and B3 and 4 µM of LF and LB.

LAMP optimization

The results of LAMP optimization showed that ladder-like pattern LAMP products were detected from the incubation at all tested temperatures consisting of 61, 63, 65 and 67°C for 60 min (Fig. 1A). Then, the time of incubation was varied, 30, 45 and 60 min at 65°C according to the recommended temperature from the manufacturer. The results revealed that LAMP products were detected from incubation at 65°C for 30, 45 and 60 min (Fig. 1B). However, at 30 min of incubation even the LAMP product was observed. The color of LAMP reaction turned orange, an incomplete reaction, which could be confused with the negative control which was pink. Incubation for 45 and 60 min gave colorimetric LAMP results, namely a color change from pink to yellow, whereas negative reactions remained pink (Figs 1A and B). Therefore, the selected optimal conditions of LAMP detection were incubation at 65°C for 45 min.

Sensitivity assay

The concentration of plasmid DNA measured by a NanoDrop machine was restricted to 1 ng · µl^{-1}. Dilutions which were lower than 1 ng · µl^{-1} were diluted continuously for 10 times of each dilution. LOD of LAMP were up to 1 fg · µl^{-1} which was 10^4 times greater than that of RT-PCR detection (Fig. 2A), whereas LOD of RT-PCR was up to 100 pg · µl^{-1} (Fig. 2C). The colorimetric observation represented the results related to gel electrophoresis (LAMP-AGE) analysis by the color of LAMP reactions from 1 µg · µl^{-1} – 1 fg · µl^{-1} which changed from pink to yellow, but the negative control (NC) and diluted plasmid DNA at 100 ag · µl^{-1} remained pink (Fig. 2B).

Specificity assay

To determine the possibility of cross reaction, LAMP was performed according to the optimal conditions using cDNA of other positive-stranded RNA viruses and viroids. Related to colorimetric results the results showed that the LAMP product was only observed in the PC lane (positive control using a plasmid DNA of CSVd) but was not found in other lanes of other different viruses and viroids consisting of CMV, CVB, TMV, TuMV and CChMVd (Fig. 3). This indicated that colorimetric LAMP was specific to only CSVd.

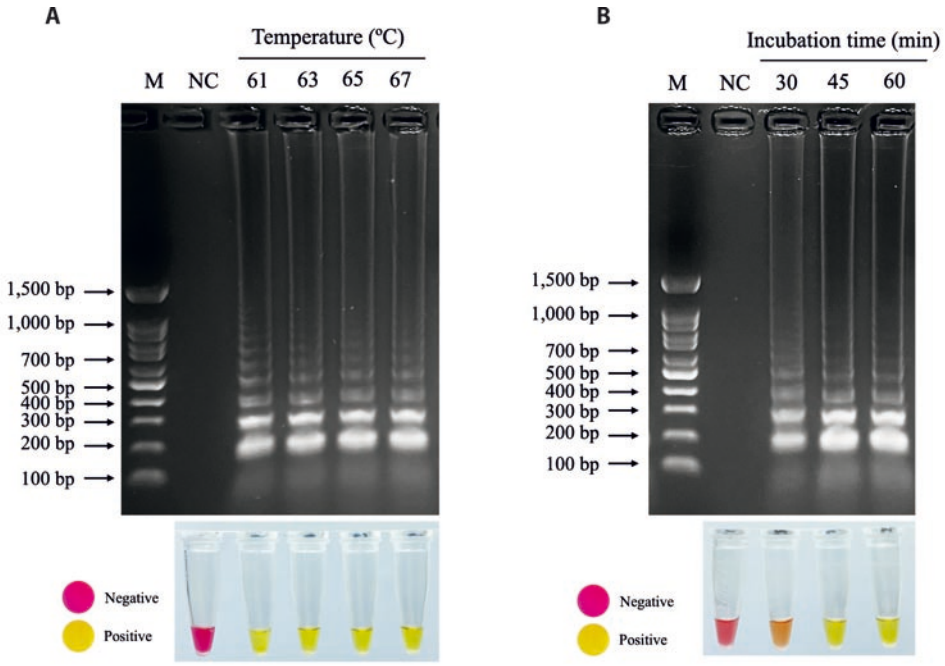

Fig. 1. Optimization of colorimetric LAMP for detecting CSVd using plasmid DNA of CSVd as a template: A – optimization of temperature at 61, 63, 65 and 67°C for 60 min; B – optimization of incubation time for 30, 45 and 60 min at 65°C. M – 100 bp + 1.5 kb DNA ladder (SibEnzyme, Russia); NC – negative control (nuclease-free water)

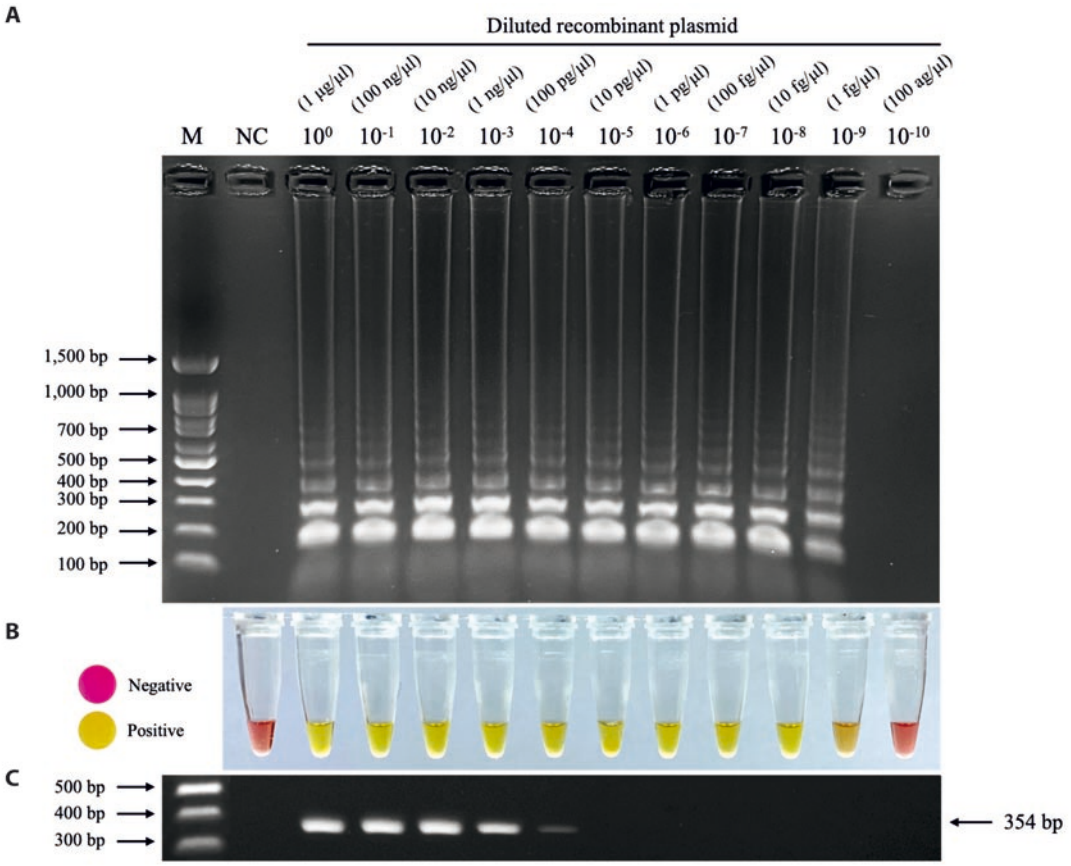

Fig. 2. Sensitivity assay of LAMP for detecting CSVd compared to PCR detection: A – LAMP-agarose gel electrophoresis (AGE) with LOD at 1 fg · μl^{-1} of diluted recombinant plasmid DNA; B – colorimetric LAMP; C – PCR with LOD at 100 pg · μl^{-1} of diluted recombinant plasmid DNA. M – 100 bp + 1.5 kb DNA ladder (SibEnzyme, Russia); NC – negative control (nuclease-free water)

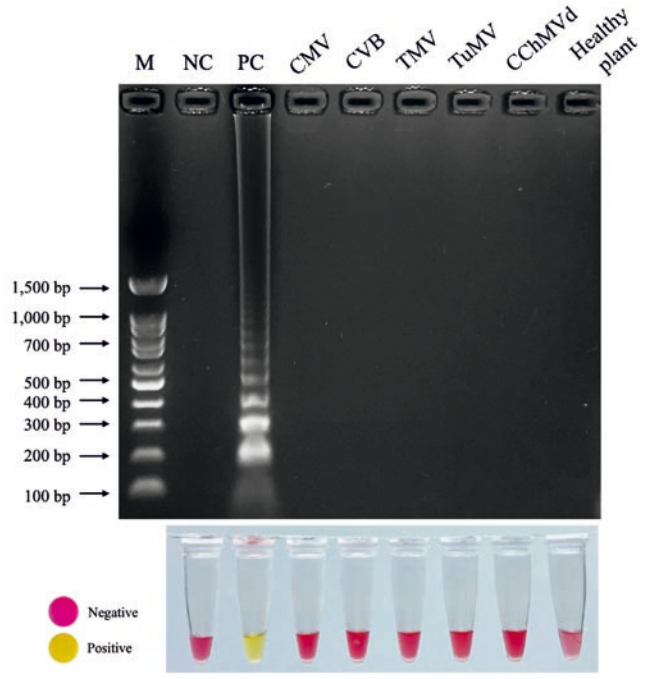

Fig. 3. Specificity assay of colorimetric LAMP for detecting CSVd. The picture shows the ladder-like pattern of LAMP products in only the PC lane (positive control using a recombinant plasmid of CSVd). CMV – cucumber mosaic virus; CVB – chrysanthemum virus B; TMV – tobacco mosaic virus; TuMV – turnip mosaic virus and CChMVd: chrysanthemum chlorotic mottle viroid; M – 100 bp + 1.5 kb DNA ladder (SibEnzyme, Russia); NC – negative control (nuclease-free water)

Application of colorimetric LAMP in detection from actual samples

To evaluate the efficiency of colorimetric LAMP for detecting CSVd, a total of 6 cDNAs from previous research (Supakitthanakorn *et al.* 2022a) was used along with cDNA which were newly synthesized from extracted RNA of 12 chrysanthemum plantlets obtained from meristem tip culture for verifying CSVd-free plantlet production (Fig. 4). The results revealed that six CSVd-positive samples were positive while 12 other samples were negative (Fig. 5A). The colorimetric assay showed the related results to LAMP-AGE by observing the change of color from pink to yellow in the

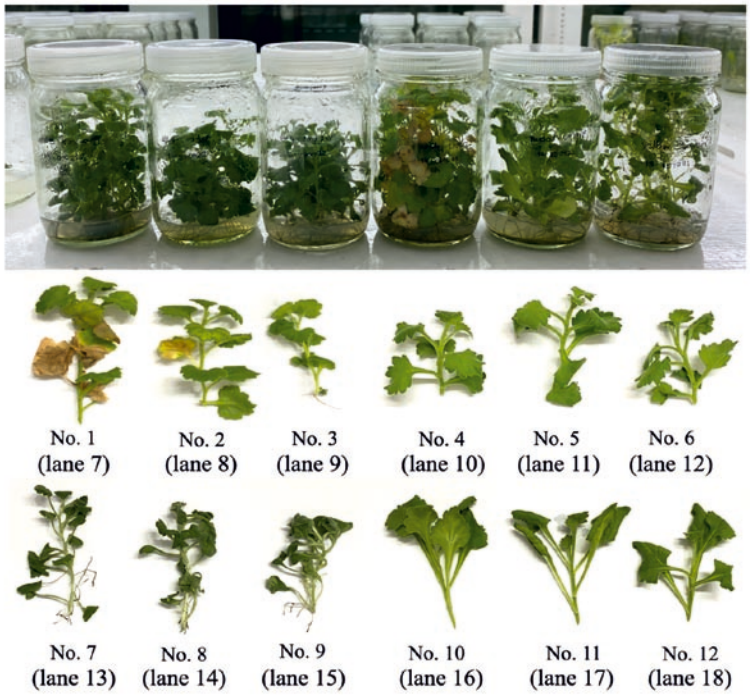

Fig. 4. Chrysanthemum plantlet samples obtained from meristem tip culture for production of virus and viroid-free plantlets which were used to evaluate the efficiency of colorimetric LAMP in detecting CSVd

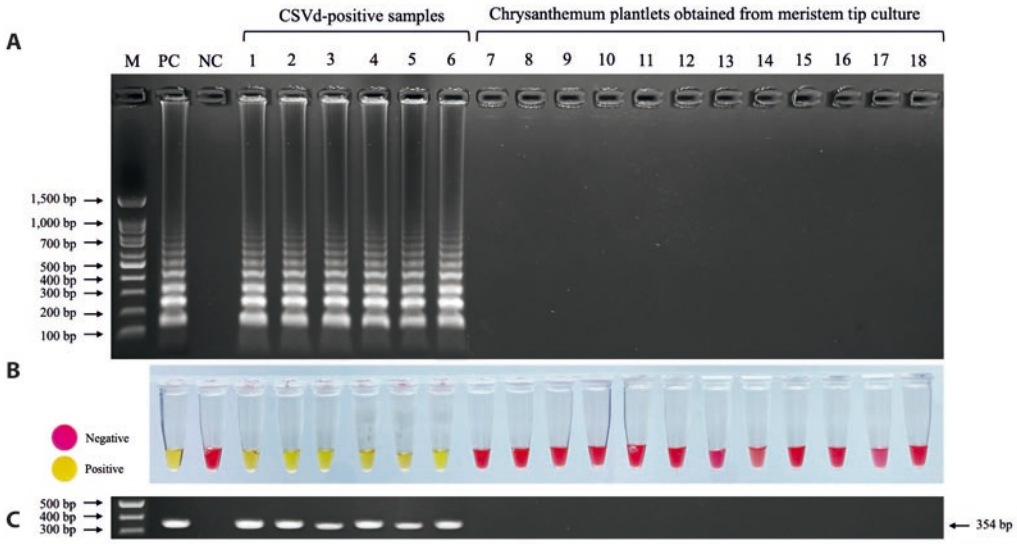

Fig. 5. Evaluation of colorimetric LAMP for detecting CSVd from actual samples: A – LAMP detection from CSVd-positive samples (lanes 1–6) showed ladder-like pattern LAMP products and 12 chrysanthemum plantlets obtained from meristem tip culture (lanes 7–19) showed negative results; B – colorimetric observation of LAMP reactions showed that the color of positive samples turned from pink to yellow; C – RT-PCR detection with 354 bp amplicons of whole genome of CSVd. M – 100 bp + 1.5 kb DNA ladder (SibEnzyme, Russia); PC – positive control (recombinant plasmid of CSVd); NC – negative control (nuclease-free water)

positive results (6 CSVd-positive samples) while negative results (12 chrysanthemum plantlets) remained pink (Fig. 5B). PCR detection was used to compare with LAMP detection and found that PCR amplicons of whole genome CSVd (354 bp) were detected from positive controls using plasmid DNA of CSVd and 6 CSVd-positive samples (Fig. 4C). A total of 12 chrysanthemum plantlet samples and negative control showed negative reactions (Fig. 4C).

Discussion

LAMP has been used to detect various plant pathogens which could be completed within 30–60 min after incubation at isothermal temperatures ranging from 60 to 68°C (Katoh *et al.* 2016; Bhuvitarkorn *et al.* 2019; Koh *et al.* 2020; Suzaki *et al.* 2022). However, it was dependent on the use of a LAMP master mix. In this study, the colorimetric LAMP was completed within 45 min after incubation at 65°C using plasmid DNA of CSVd as a template. Under these conditions, LAMP products were detected and colorimetric results were observed. The colorimetric results of positive and negative reactions were easily distinguished by visualization of a color change from pink to yellow in a positive result which did not require any additional instruments such as UV or fluorescent lamps for result analysis.

A RT-LAMP for detecting CSVd was previously developed by Liu *et al.* (2014). The previous RT-LAMP technique could detect CSVd and CVB simultaneously from chrysanthemum plants. This study represented a recent alternative way to visualize LAMP reaction by observation of a color change, known as colorimetric for eliminating laborious post-amplification work by enhancing the rapidness of detection and the results clearly distinguished between positive and negative results.

Colorimetric LAMP showed detection results directly by changing from pink to yellow, using phenol red as an indicator dye (WarmStart® Colorimetric LAMP 2X Master Mix with UDG, New England Biolabs, USA) which was due to the change of pH in the reaction (Scott *et al.* 2020). LAMP reactions can produce a 2–3 pH unit drop without loss of reaction efficiency. This pH change is sufficient to use neutral-range pH sensitive indicator dyes, such as phenol red and neutral red (Tanner *et al.* 2015). Phenol red-based LAMP provides a clear distinction between positive and negative samples unlike many other metal-sensitive indicators that can be difficult to visually distinguish (Poole *et al.* 2017).

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; Nebeshima *et al.* 2017). RT-PCR with low LOD, sometimes, could not detect the presence of viroids (Liu *et al.* 2014). This study conducted the colorimetric LAMP for detection of CSVd in chrysanthemum plantlets which were obtained from meristem tip culture. The results showed that the developed colorimetric LAMP could be used to detect CSVd effectively from positive CSVd chrysanthemum samples whereas all chrysanthemum plantlets were negative. In conclusion, the colorimetric LAMP that was developed in this study had the potential effectiveness of detecting CSVd in chrysanthemum due to its sensitivity, specificity and convenience and could be used routinely to detect CSVd from field samples. It could also be used to verify and ensure the production of CSVd-free chrysanthemum plantlets.

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References

Almasi M.A. 2017. Development of a colorimetric reverse transcription loop-mediated isothermal amplification assay for the detection of mirafiori lettuce big-vein virus. Archives of Virology 162 (9): 2775–2880. DOI: http://doi.org/10.1007/s00705-017-3404-3

Bester R., Maree H.J. 2022. A reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for the detection of plum viroid I (PlVd-I). Journal of Virological Methods 306: 114543. DOI: https://doi.org/10.1016/j.jviromet.2022.114543

Bhuvitarkorn S., Klinkong S., Reanwarakorn K. 2019. Enhancing columnea latent viroid detection using reverse transcription loop-mediated isothermal amplification (RT-LAMP). International Journal of Agricultural Technology 15 (2): 215–228.

Camps R., Mansur L., De la Cuadra C., Besoain X. 2015. First report of turnip mosaic virus (TuMV) infecting *Leucocoryne purpurea* (Amaryllidaceae) in Chile. Plant Disease 100 (11): 2341. DOI: https://doi.org/10.1094/PDIS-05-16-0777-PDN

Chen S., Gu H., Wang X., Chen J., Zhu W. 2011. Multiplex RT--PCR detection of cucumber mosaic virus subgroups and tobamoviruses infecting tomato using 18S rRNA as an internal control. Acta Biochimica et Biophysica Sinica 43: 465–471. DOI: https://doi.org/ 10.1093/abbs/gmr031

Cho W.K., Jo Y., Jo K.M., Kim K.H. 2013. A current overview of two viroids that infect chrysanthemums: chrysanthemum stunt viroid and chrysanthemum chlorotic mottle viroid. Viruses 5: 1099–1113. DOI: https://doi.org/10.3390/v5041099

Chung B.N., Huh E.J., Kim J.S. 2006. Effect of temperature on the concentration of chrysanthemum stunt viroid in CSVd-infected chrysanthemum. The Plant Pathology Journal 22 (2): 152–154. DOI: https://doi.org/10.3389/fmicb.2016.00224

Di Serio F., Flores R. 2008. Viroids: molecular implements for dissecting RNA trafficking in plants. RNA Biology 5: 128–131. DOI: https://doi.org/10.4161/rna.5.3.6638

Ding B., Itaya A. 2007. Viroids: a useful model for studying the basic principle of infection and RNA biology. Molecular Plant-Microbe Interaction 20: 7–20. DOI: https://doi.org/10.1094/MPMI-20-0007

Doi M., Kato K. 2004. Nucleotide sequence of chrysanthemum stunt viroid (CSVd) occurred in Shizuoka Prefecture and symptoms of chrysanthemum cultivar. Annual Report of The Kansai Plant Protection Society 46: 11–14.

Ebata M., Matsushita Y., Morimoto M., Mochizuki T. 2019. Distribution of chrysanthemum chlorotic mottle viroid in shoot meristem and flower buds of chrysanthemum. European Journal of Plant Pathology 154: 555–563. DOI: https://doi.org/10.1007/s10658-019-01679-1

Fukuta S., Ohishi K., Yoshida K., Mizukami Y., Ishida A., Kanbe M. 2004. Development of immunocapture reverse transcription loop-mediated isothermal amplification for the detection of tomato spotted wilt virus from chrysanthemum. Journal of Virological Methods 121 (1): 49–55. DOI: https://doi.org/10.1016/j.jviromet.2004.05.016

Gobatto D., de Oliveira L.A., Franco D.A.S., Velasquez N., Daros J.A., Eiras M. 2019. Surveys in the chrysanthemum production areas of Brazil and Colombia reveal that weeds are potential reservoirs of chrysanthemum stunt viroid. Viruses 11 (4): 355. DOI: https://doi.org/10.3390/v11040355

Guan Z., Wu D., Song A., Chen F., Chen S., Fang W. 2017. A highly sensitive method for the detection of chrysanthemum virus B. Electronic Journal of Biotechnology 26: 64–68. DOI: https://doi.org/10.1016/j.ejbt.2017.01.003

Hasiów-Jaroszewska B., Borodynko N. 2013. Detection of Pepino mosaic virus isolates from tomato by one-step reverse transcription loop-mediated isothermal amplification. Archives of Virology 158 (10): 2153–2156. DOI: https://doi.org/10.1007/s00705-013-1706-7

Haseloff J., Symons R. 1981. Chrysanthemum stunt viroid: primary sequence and secondary structure. Nucleic Acids Research 9 (12): 2741–2752. DOI: https://doi.org/10.1093/nar/9.12.2741

Horst R.K., Langhans R.W., Smith S.H. 1977. Effects of chrysanthemum stunt, chlorotic mottle, aspermy and mosaic on flowering and rotting of chrysanthemums. Phytopathology 67: 9–14.

Hosokawa M., Otake A., Ohishi K., Ueda E., Hayashi T., Yazawa S. 2004. Elimination of chrysanthemum stunt viroid from an infected chrysanthemum cultivar by shoot regeneration from a leaf primordium-free shoot apical meristem dome attached to a root tip. Plant Cell Reports 22 (11): 859–863. DOI: https://doi.org/10.1007/s00299-004-0770-6

Jeong J.J., Ju H.J., Noh J. 2014. A review of detection methods for the plant viruses. Research in Plant Disease 20 (3): 173–181. DOI: http://dx.doi.org/10.5423/RPD.2014.20.3.173

Katoh H., Fukuda T., Nishigawa H., Natsuaki T. 2016. Rapid detection of *Colletotrichum gloeosporioides* in infected strawberry plants using loop-mediated isothermal amplification. Journal of General Plant Pathology 82 (4): 190–198. DOI: https://doi.org/10.1007/s10327-016-0665-8

Koh R.B.L., Barbosa C.F.C., Aquino V.M., Galvez L.C. 2020. Rapid, simple detection of banana bract mosaic virus in abaca using a one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay. Journal of General Plant Pathology 86: 433–441. DOI: https://doi.org/10.1007/s10327-020-00949-9

Kumar S., Shankar A.S.U., Nayaka S.C., Lund O.S., Prakash H.S. 2011. Detection of tobacco mosaic virus and tomato mosaic virus in pepper and tomato by multiplex RT-PCR. Letters in Applied Microbiology 53 (3): 359–363. DOI: https://doi.org/10.1111/j.1472-765X.2011.03117.x

Liu X.L., Zhao X.T., Muhammad I., Ge B.B., Hong B. 2014. Multiplex reverse transcription loop-mediated isothermal amplification for the simultaneous detection of CVB and CSVd in chrysanthemum. Journal of Virological Methods 210: 26–31. DOI: https://doi.org/10.1016/j.jviromet.2014.09.008

Liu Z., Xia Z., Yang C., Huang J. 2016. Colorimetric detection of maize chlorotic mottle virus by reverse transcription loop-mediated isothermal amplification (RT-LAMP) with hydroxynapthol blue dye. RSC Advances 6: 73–78. DOI: https://doi.org/10.1039/ C5RA20789D

Matsushita Y. 2013. Chrysanthemum stunt viroid. Japan Agricultural Research Quarterly 47 (3): 237–242. DOI: https://doi.org/10.6090/jarq.47.237

Matsushita Y., Yanagisawa H., Khiutti A., Mironenko N., Ohto Y., Afanasenko O. 2019. First report of chrysanthemum stunt viroid variant from potato (*Solanum tuberosum*) plants in Russia. Journal of General Plant Pathology 85 (4): 311–313. DOI: https://doi.org/10.1007/s10327-019-00851-z

Nabeshima T., Doi M., Hosokawa M. 2017. Comparative analysis of chrysanthemum stunt viroid accumulation and movement in two chrysanthemum (*Chrysanthemum morifolium*) cultivars with differential susceptibility to the viroid infection. Frontiers in Plant Science 8: 1940. DOI: https://doi.org/10.3389/fpls.2017.01940

Notomi T., Okayama H., Masubuchi H., Yonekawa T., Watanabe K., Amino N., Hase T. 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Research 28 (12): e63. DOI: https://doi.org/10.1093/nar/28.12.e63

Panno S., Matic S., Tiberini A., Caruso A.G., Bella P., Torta L., Stassi R., Davino S. 2020. Loop mediated isothermal amplification: Principles and applications in plant virology. Plants 9 (4): 461. DOI: https://doi.org/10.3390/plants9040461

Poole C.B., Li Z., Alhassan A., Guelig D., Diesburg S., Tanner N.A., Zhang Y., Evans Jr.T.C., LaBarre P., Wanji S., Burton R.A., Carlow C.K.S. 2017. Colorimetric tests for diagnosis of filarial infection and vector surveillance using non-instrumented nucleic acid loop-mediated isothermal amplification (NINA-LAMP). PLoS ONE. DOI: https://doi.org/10.1371/journal.pone.0169011

Scott A.T., Layne T.R., O'Connell K.C., Tanner N.A., Landers J.P. 020. Comparative evaluation and quantitative analysis of loop-mediated isothermal amplification indicators. Analytical Chemistry 92 (19): 13343–13353. DOI: https://doi.org/10.1021/acs.analchem.0c02666

Supakitthanakorn S., Vichittragoontavorn K., Kunasakdakul K., Ruangwong O. 2022a. 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.

Supakitthanakorn S., Vichittragoontavorn K., Kunasakdakul K., Thapanapongworakul P., Sunpapao A., Ruangwong O. 2022b. Tobacco mosaic virus infection of chrysanthemums in Thailand: development of colorimetric reverse-transcription loop-mediated isothermal amplification (RT-LAMP) technique for sensitive and rapid detection. Plants 11: 1788. DOI: https://doi.org/10.3390/plants11141788

Suzaki K., Sawada H., Kisaki G. 2022. Loop-mediated isothermal amplification of bacterial effector genes to detect *Pseudomonas syringae* pv. *actinidiae* biovars 1 and 3. Journal of General Plant Pathology 88 (1): 2–9. DOI: https://doi.org/10.1007/s10327-021-01030-9

Tanner N.A., Zhang Y., Evans Jr.T.C. 2015. Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes. Biotechniques 58 (2): 59–68. DOI: https://doi.org/10.2144/000114253

Verma N., Ram R., Hallan V., Kumar K., Zaidi A.A. 2004. Production of cucumber mosaic virus-free chrysanthemums by meristem tip culture. Journal of Crop Protection 23 (5): 469–473. DOI: https://doi.org/10.1016/j.cropro.2003.08.021

Zalewska M., Lema-Ruminska J., Miler N. 2007. *In vitro* propagation using adventitious buds technique as a source of new variability in chrysanthemum. Scientia Horticulturae 113 (1): 70–73. DOI: https://doi.org/10.1016/j.scienta.2007.01.019

Supplementary information

Table S1. PCR primers used for detection of viruses and CChMVd in the specificity assay

Virus/viroid	Primer name	Nucleotide sequence (5′-3′)	Ta [°C]	Product size [bp]	Reference
CMV	CMV-I-F CMV-I-R	GCCACCAAAAATAGACCG ATCTGCTGGCGTGGATTTCT	52	593	Chen *et al.* (2011)
CVB	CVB-F1 CVB-R1	AGTCACAATGCCTCCCAAAC CATACCTTTCTTAGAGTGCTATGCT	53	621	Guan *et al.* (2017)
TuMV	TuMV-F TuMV-R	CAAGCAATCTTTGAGGATTATG TATTTCCCATAAGCGAGAATA	56	986	Camps *et al.* (2015)
TMV	TMV-F TMV-R	CGACATCAGCCGATGCAGC ACCGTTTTCGAACCGAGACT	60	880	Kumar *et al.* (2011)
CChMVd	CCh-nF CCh-MR1	CAGGATCGAAACCTCTTCCA GACCTCTTGGGGGTTAGAAA	59	398	Ebata *et al.* (2019)

Ta – annealing temperature; CMV – cucumber mosaic virus, CVB – chrysanthemum virus; TuMV – turnip mosaic virus; TMV – tobbaco mosaic virus; CChMVd – chrysanthemum chlorotic mottle viroid