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First characterisation of chrysanthemum virus B infecting chrysanthemum in Thailand and development of colourimetric RT-LAMP for rapid and sensitive detection

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ABSTRACT

Chrysanthemum is among the world's most important ornamental plants because of its high economic and cultural value. Our report is the first to describe the detection of chrysanthemum virus B (CVB) in chrysanthemum leaf samples collected from Thailand, which showed yellowing and mild mottling symptoms. The coat protein sequences of CVB isolated in this study share 95.15% identity with previously characterised CVB isolates. Biological indexing found that CVB induced both local and systemic symptoms in tobacco plants, while petunia displayed systemic symptoms. To improve the rapidity and sensitivity of CVB detection, the loop-mediated isothermal amplification (LAMP) technique was developed. LAMP detection was found to be optimal when incubation was conducted at 65 °C for 45 min, wherein the LAMP reaction demonstrated 10^6 times higher sensitivity than polymerase chain reaction. To simplify the interpretation of results, we designed the method such that a positive result is clearly indicated based on a change of colour (colourimetry), from pink to yellow, as observed visually and via gel electrophoresis. To our best knowledge, this is the first report on the characterisation of molecular, biological and morphological characteristics of CVB infecting chrysanthemum in Thailand, along with the development of colourimetric RT-LAMP for improving detection efficiency.

Keywords: ornamental plant, rapid detection, virus detection, virus disease, virus particle

Abbreviations: CVB, chrysanthemum virus B; LAMP, loop-mediated isothermal amplification; RT-PCR, reverse transcription polymerase chain reaction; SDT, sequence demarcation tool.

INTRODUCTION

Chrysanthemum (*Chrysanthemum* × *morifolium*) is a commercially important ornamental plant grown as flowers for cutting and pot plants (Da Silva and Kulus, 2014). Virus and viroid diseases are major limiting factors in chrysanthemum cultivation throughout the world. Numerous viruses and viroids are known to infect chrysanthemum, including chrysanthemum virus B (CVB), cucumber mosaic virus (CMV), chrysanthemum

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stem necrosis virus (CSNV), impatiens necrotic spot virus (INSV), tobacco mosaic virus (TMV), tomato aspermy virus (TAV), tomato spotted wilt virus (TSWV), zucchini yellow mosaic virus (ZYMV), chrysanthemum chlorotic mottle viroid (CChMVd) and chrysanthemum stunt viroid (CSVd) (Raizada et al., 1989; O'Reilly et al., 1991; Martelik and Mokra, 1998; Kondo et al., 2011; Song et al., 2012; Niu et al., 2015; Zhao et al., 2015). Traditionally, chrysanthemums are mainly propagated through cuttings, which are highly conductive to the accumulation and spread of virus and viroid diseases (Liu et al., 2014). To eradicate virus and viroid contamination in propagated cuttings, the meristem tissue culture technique has been used to produce virus-free chrysanthemum plantlets (Da Silva and Kulus, 2014).

Chrysanthemum virus B was found to be the most frequent chrysanthemum-infecting virus, followed by CMV, TAV and members of *Tospovirus* and *Potyvirus* (Verma et al., 2003). Most commercial chrysanthemum cultivars are usually infected with CVB without showing visible symptoms. Chrysanthemum plants infected with CVB show different symptoms depending on the cultivar, such as mosaic, mottling, vein clearing, chlorosis, necrotic streaks, malformation and corrugation of the leaf blade (Hollings, 1957; Lin et al., 2005; Ohkawa et al., 2008). CVB, formerly known as chrysanthemum mild mosaic virus, is a member of the genus *Carlavirus* in the family Betaflexiviridae (Singh et al., 2007). CVB has slightly flexuous rod-like particles (650–685 nm long and 12 nm wide) without a protein envelope. The genome of CVB is a single linear, positive-sense, single-stranded RNA (+ssRNA) with 8,000–9,000 nucleotides excluding the poly(A) tail, and the genome contains six open reading frames (ORFs) (Singh et al., 2012). Transmission of CVB occurs via aphids *Myzus persicae* and *Aphis gossypii* in a non-persistent manner and also through sap (Ohkawa et al., 2007).

Chrysanthemum virus B is globally distributed according to where chrysanthemums are grown and has been reported in many countries of Asia, including India (Verma et al., 2004), Japan (Yamamoto et al., 2001), Taiwan (Lin et al., 2005) and China (Zhao et al., 2015). In Thailand, chrysanthemums have been grown for decades, but the detection of CVB has not been reported.

Molecular techniques based on polymerase chain reaction (PCR) are commonly used to diagnose plant virus diseases. Loop-mediated isothermal amplification (LAMP) has been widely applied for the detection of many plant viruses and can detect both DNA and RNA viruses (reverse transcription (RT)-LAMP) (Panno et al., 2020). The LAMP technique was first developed by Notomi et al. (2000), and it has high sensitivity and specificity based on the use of four specific primers that recognise six distinct regions on the target DNA. LAMP reactions require four to six primers (loop-F and loop-B are additional primers) that are specific to six to eight positions on the target gene, leading to higher sensitivity and specificity compared to reverse transcription polymerase chain reaction (RT-PCR), which requires only two primers (Panno et al., 2020). The LAMP reaction is based on DNA strand displacement activity mediated by *Bst* polymerase from *Geobacillus stearothermophilus* under isothermal conditions, which enables the use of an inexpensive heat block or water bath incubator, which is appropriate for field detection.

In this study, we aimed to detect and identify CVB infecting chrysanthemum in Thailand and describe the molecular, biological and structural characteristics of the Thai CVB isolate. To improve the effectiveness of virus detection, colourimetric RT-LAMP was developed.

MATERIALS AND METHODS

Sample collection

Surveys were conducted at chrysanthemum plantations in Chiang Mai and Chiang Rai Provinces, northern Thailand, during 2019–2021. Chrysanthemum leaves showing virus-like symptoms, such as mild mottling, vein clearing and yellowing, in addition to samples without symptoms, were collected for detection of CVB by RT-PCR. The percentage of disease incidence (PDI) was calculated as described by Ali et al. (2013).

RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from 0.1 g chrysanthemum leaf using TRIzol® Reagent (Invitrogen, Waltham, USA) according to the manufacturer's instructions. The RT reaction was performed by using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) following the manufacturer's protocol for synthesising cDNA. The cDNA was kept at −20 °C until use in PCR and colourimetric LAMP detection.

RT-PCR detection

The primer set specific to partial triple block gene-3 (*TBG3*) (ORF 4) and partial coat protein (*CP*) gene (ORF5) was used for CVB detection, and the primer set for amplification of whole *CP* gene was used for sequencing (Table 1). PCR reactions were performed by using EconoTaq® PLUS & PLUS GREEN 2× Master Mixes (Lucigen, Wisconsin, USA) in the DNA Engine® Peltier Thermal Cycler PTC-200 (Bio-Rad, Hercules, USA). The PCR reaction was performed as follows: one cycle of initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54/56 °C for 30 s, extension at 72 °C for 45 s and one cycle of final extension at 72 °C for 7 min. PCR products were visualised on 1.5% agarose gel electrophoresis (AGE) stained with RedSafe™ Nucleic Acid Staining Solution (iNtRON, Gyeonggi, Korea) in the MIL-DUT48 blue light transilluminator (MIULAB Instruments, China). The size of PCR products was compared to GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher, Waltham, USA).

Table 1. Primers used for detection of CVB by RT-PCR and colourimetric RT-LAMP techniques in this study.

Type of detection	Primer name	Sequence (5'-3')	Target gene(s)	Ta*** (°C)	Reference
PCR	CVB-F_1	AGTCACAATGCCTCCCAAAC	*TGB3*** + *CP*	54	Guan et al. (2017)
	CVB-R_1	CATACCTTTCTTAGAGTGCTATGCT			
PCR and sequencing	CVB-up	TAGGTTGTGGAGTGGTTACA	*CP*	56	Lin et al. (2005)
	CVB-dw	ATCTTCACAATGACATCCAT			
LAMP	FIP*	CCTGCTCACGCTCTCGTTCCCAGCTCGAACAGCGGAAG	*TGB3* + *CP*	63–68	This study
	BIP	AGATGAACTCCAATGCCCCAGCTAGTGCCGCGAGTTGTGT			
	F3	ACCAACTCCACCTCCACC			
	B3	TCGTCTTGCTCTCTCCTCAG			

*FIP: forward inner primer containing F1c + F2.
**_TGB3_: triple gene block 3.
***Ta: annealing temperature.
BIP, backward inner primer containing B1c + B2; B3, backward primer; _CP_, coat protein gene; CVB, chrysanthemum virus B; F3, forward primer; LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction.

Cloning, sequencing and phylogenetic tree analysis

The PCR products of partial *TGB3* and *CP* genes were purified using PCR Clean-Up and Gel Extraction Kits (Bio-Helix, New Taipei City, Taiwan) according to the manufacturer's instructions. Purified PCR products were ligated into pCR®2.1-TOPO® vector (Invitrogen) and transformed into TOP10TM chemically competent Escherichia coli cells (Invitrogen) by heat shock transformation according to the manufacturer's instructions. The positive clones were selected and subsequently sequenced. Nucleotides were sequenced by fluorescent dye-terminator sequencing on an ABI Prism™ 3730xl DNA sequencer (Applied Biosystems, Foster City, CA, USA). The obtained sequences (NCBI accession numbers: OL804013 and OL804014) were aligned and analysed using Molecular Evolutionary Genetics Analysis (MEGA) X software (Kumar et al., 2018) and BLAST, respectively. In the case of any inconsistency, at least two more clones were sequenced to obtain the consensus sequence. The percentage identity matrix was calculated using the sequence demarcation tool (SDT) version 1.2 (Muhire et al., 2014) and compared with previous CVB isolates and other members of the genus *Carlavirus* in GenBank. Multiple alignment of CVB *CP* sequences was performed in CLC Sequence Viewer version 8.0 (Qiagen, Maryland, USA). The phylogenetic tree was constructed in MEGA X using the maximum likelihood (ML) method with 1,000 bootstrap replicates.

Bioassay on indicator plants

To study the pathogenicity of CVB in indicator plants, the virus was isolated from infected chrysanthemum leaves that were determined to be CVB positive based on RT-PCR. The leaves were ground in 0.1 M phosphate buffer (pH 7.0) and then mechanically inoculated with *Chenopodium quinoa* for local lesion isolation, with subsequent inoculation of chrysanthemum seedlings, *C. amaranticolor*, *Nicotiana tabacum* cv. *Xanthi*, *N. tabacum* cv. *Samsun*, *N. benthamiana*, *N. glutinosa*, *Vigna unguiculata* and petunia (*Petunia × hybrida*). The plants were kept in the greenhouse at 25–28 °C. RT-PCR was used for confirmation of virus infection.

LAMP primers design and optimisation of LAMP reaction

Four LAMP primers, consisting of forward inner primer (FIP), backward inner primer (BIP), forward primer (F3) and backward primer (B3), were designed based on the partial *TGB3* and *CP* gene of CVB (accession no. EU499736.1) retrieved from GenBank using PrimerExplorer V.5 software (Eiken, Japan) with the default setting.

Colourimetric LAMP reaction was performed by using WarmStart® Colourimetric LAMP 2× Master Mix with uracil DNA glycosylase (UDG) (New England Biolabs, Ipswich, USA). A 25.0 µL aliquot of

LAMP component was added to the 200 µL PCR tube containing 12.5 µL WarmStart® Colourimetric LAMP 2× Master Mix with UDG, 2.5 µL LAMP Primer Mix (10×), 1.0 µL of recombinant plasmid DNA and 9.0 µL of nuclease-free H_2O. LAMP was performed at different incubation temperatures of 60 °C, 63 °C, 65 °C and 68 °C for 60 min to determine the optimal incubation temperature in terms of sensitivity. Subsequently, the incubation time was determined at the selected temperature for 30 min, 45 min and 60 min. The LAMP products were analysed using 1.5% AGE in 1× TBE buffer, and the gel was stained with RedSafe™ Nucleic Acid Staining Solution (iNiTron). Moreover, the results are colourimetric, whereby a change in the colour of the LAMP reaction after incubation from pink to yellow is considered a positive result, whereas a reaction that remains pink indicates a negative result.

Sensitivity assay

To evaluate the limit of detection (LOD) of the LAMP reaction, 10-fold serial dilution (10^0–10^{-10}) was performed to dilute the CVB plasmid of partial *TGB3* and *CP* genes, and the plasmid DNA solution was then quantified in the NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher, Waltham, USA). The LAMP reaction was performed under optimal conditions, and LAMP products were visualised using 1.5% AGE along with the colourimetric observation as previously described.

Specificity assay and evaluation of colourimetric RT-LAMP performance

To address possible cross-reactivity, the positive cDNA of other viruses and viroids (TMV, turnip mosaic virus (TuMV), melon yellow spot virus (MYSV), CChMVd and CSVd) and cDNA from a healthy chrysanthemum were used. The LAMP reaction was performed under optimal conditions, and LAMP products were visualised along with the colourimetric observation as previously described. To evaluate the performance of the colourimetric RT-LAMP technique in detecting CVB, 10 new chrysanthemum leaves were collected and subjected to CVB detection.

RESULTS

RT-PCR detection

Four chrysanthemum plantation areas were surveyed, and 110 samples of chrysanthemum leaf were collected. From a total of 110 samples, 95 samples displayed virus-like symptoms including mosaic, mottling, leaf malformation, chlorotic spots and necrotic spots (NSs), and 15 samples were symptomless. PCR products of 621 bp specific to the partial *TGB3* and *CP* genes of CVB were detected from two samples from the same plantation area in Chiang Mai Province (BW-54 and HL4-70) that exhibited chlorosis, yellowing and mild mottling symptoms (Figure 1). The CVB infection rate was 1.81%.

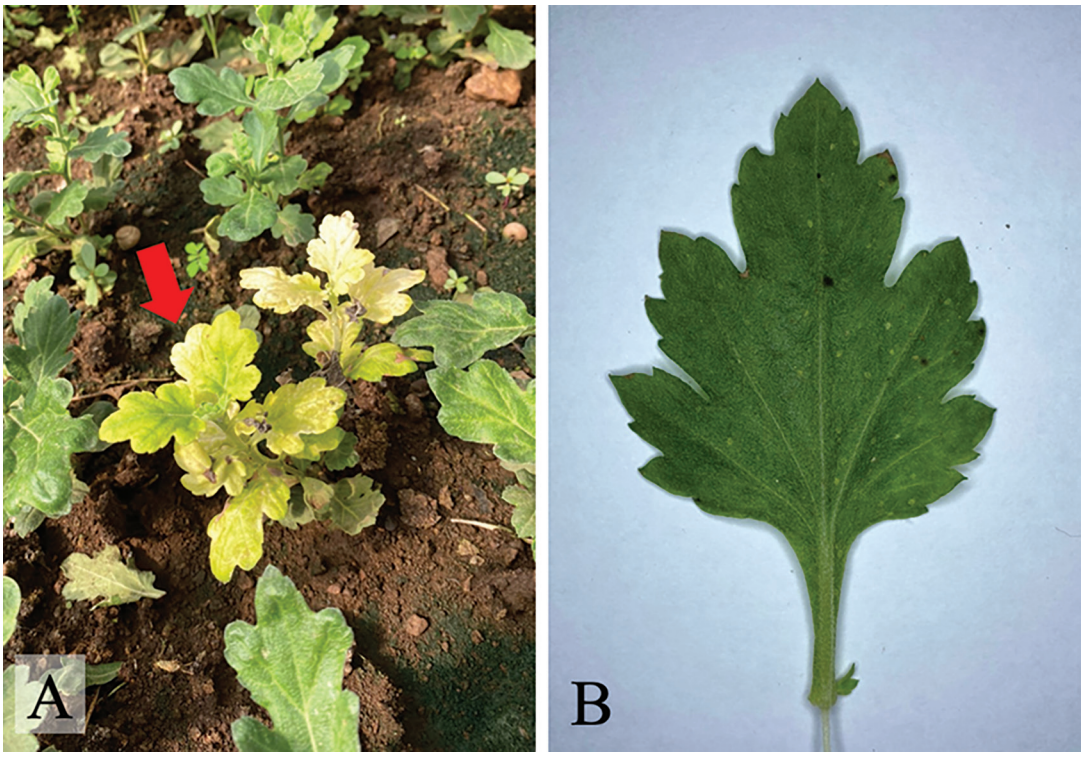

Figure 1. Symptoms observed on chrysanthemum leaves collected from Chiang Mai Province of Thailand that were positive to CVB detection by using RT-PCR. (A) Chlorosis and yellowing symptoms (the red arrow). (B) The mild mottling symptom. CVB, chrysanthemum virus B; RT-PCR; reverse transcription polymerase chain reaction.

Sequence and phylogenetic tree analysis

The complete *CP* gene sequences of CVB isolates in this study (OL804013 and OL804014) were 100% identical. Since the homology of complete *CP* gene sequences from two CVBs indicated 100% identity, we assumed that these CVBs were the same isolate from different samples. This CVB isolate shares 95.15% identity with previously available CVB isolates retrieved from GenBank, including 16 isolates from India, 3 isolates from China and 1 isolate from Russia.

The pairwise identity matrix of nucleotide sequences of CVB and other members of the genus *Carlavirus* was calculated using SDT v1.2 software (Figure 2). The matrix showed that the sequence identity among CVB isolates ranges from 83% to 100% based on the colour-coded score, and between other members of the genus *Carlavirus* it ranges from 49% to 72%.

The phylogenetic tree based on the ML method showed that CVB-BW-54 (accession №. OL804013) and CVB-HL4-70 (accession №. OL804014) clustered into the CVB clade and closely clustered with isolates from India, but far from isolates from China and Russia and some from India (Figure 3). CVB isolates are clearly separated from other members of the genus *Carlavirus*, including red clover carlavirus (RCV), ligustrum virus A (LVA), rose virus A (RVA), rose virus B, garlic latent virus (GLV), carnation latent virus (CLV), cowpea mild mottle virus (CMMoV), potato virus M (PVM), potato virus H (PVH), blueberry scorch virus (BSV), potato rough dwarf virus (PRDV) and potato virus S (PVS) (Figure 3).

Bioassay on indicator plants

CVB-BW-54 was used for the bioassay. At 10 days post-inoculation (dpi), inoculation with *C. amaranticolor* and *C. quinoa* produced chlorotic spots on inoculated leaves. CVB induced local NSs on inoculated leaves of *N. glutinosa* and *N. tabacum* cv. *Samsun* at 3 dpi (Table 2). Systemic infections were observed on

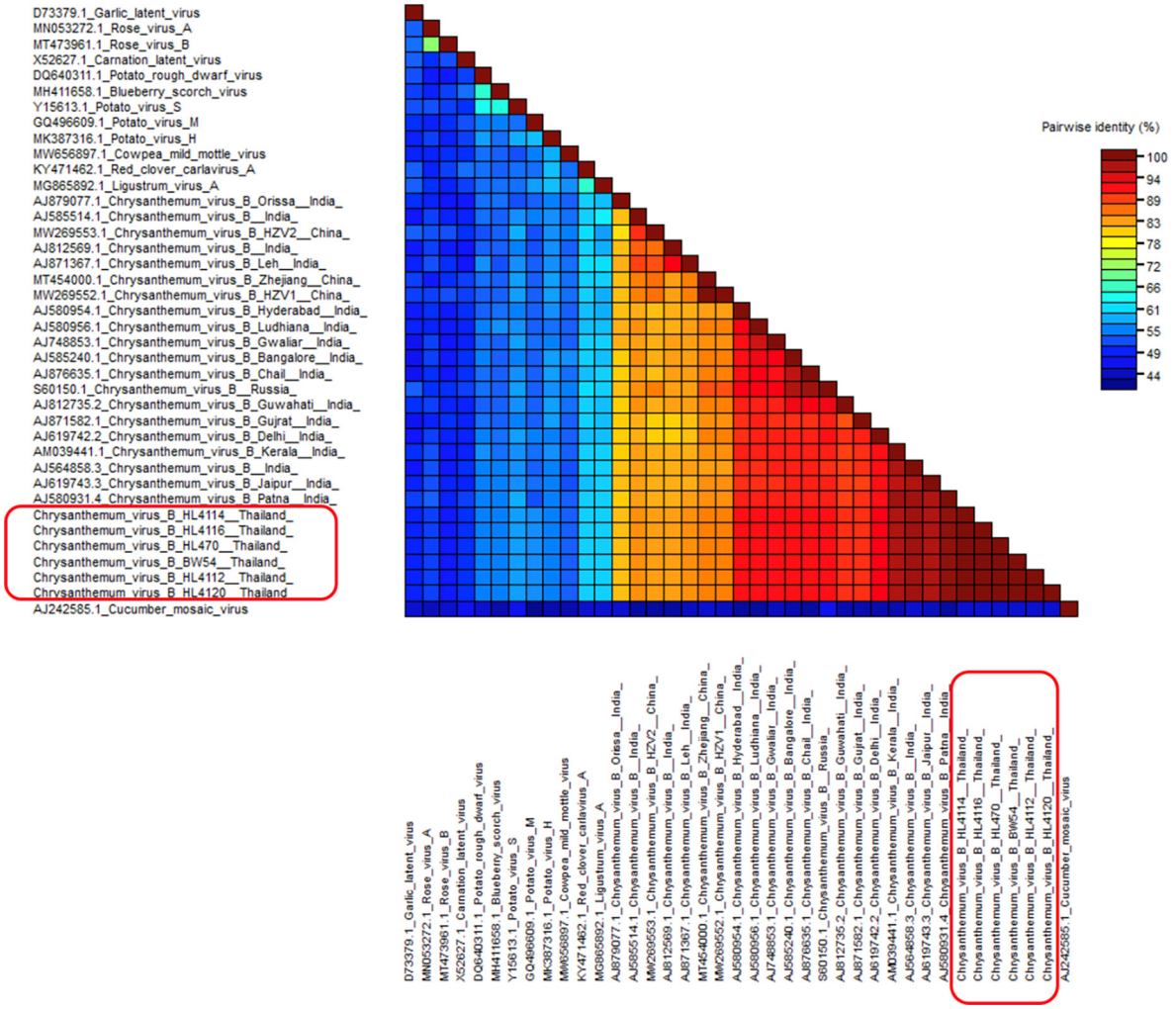

Figure 2. Colour-coded pairwise identity matrix generated from 35 virus sequences consisting of CVB isolates, other members of the genus *Carlavirus* and CMV of the genus *Cucumovirus* that was used as the outgroup. CVB isolates in this study were indicated by red boxes. The pairwise identity matrix was performed in the SDT version 1.2 program. The colour of the cell indicates the percentage identity score between two sequences (left and bottom axis) according to the colour-coded cells mean to each cell in the SDT matrix of the Figure 2. CMV, cucumber mosaic virus; CVB, chrysanthemum virus B; SDT, sequence demarcation tool.

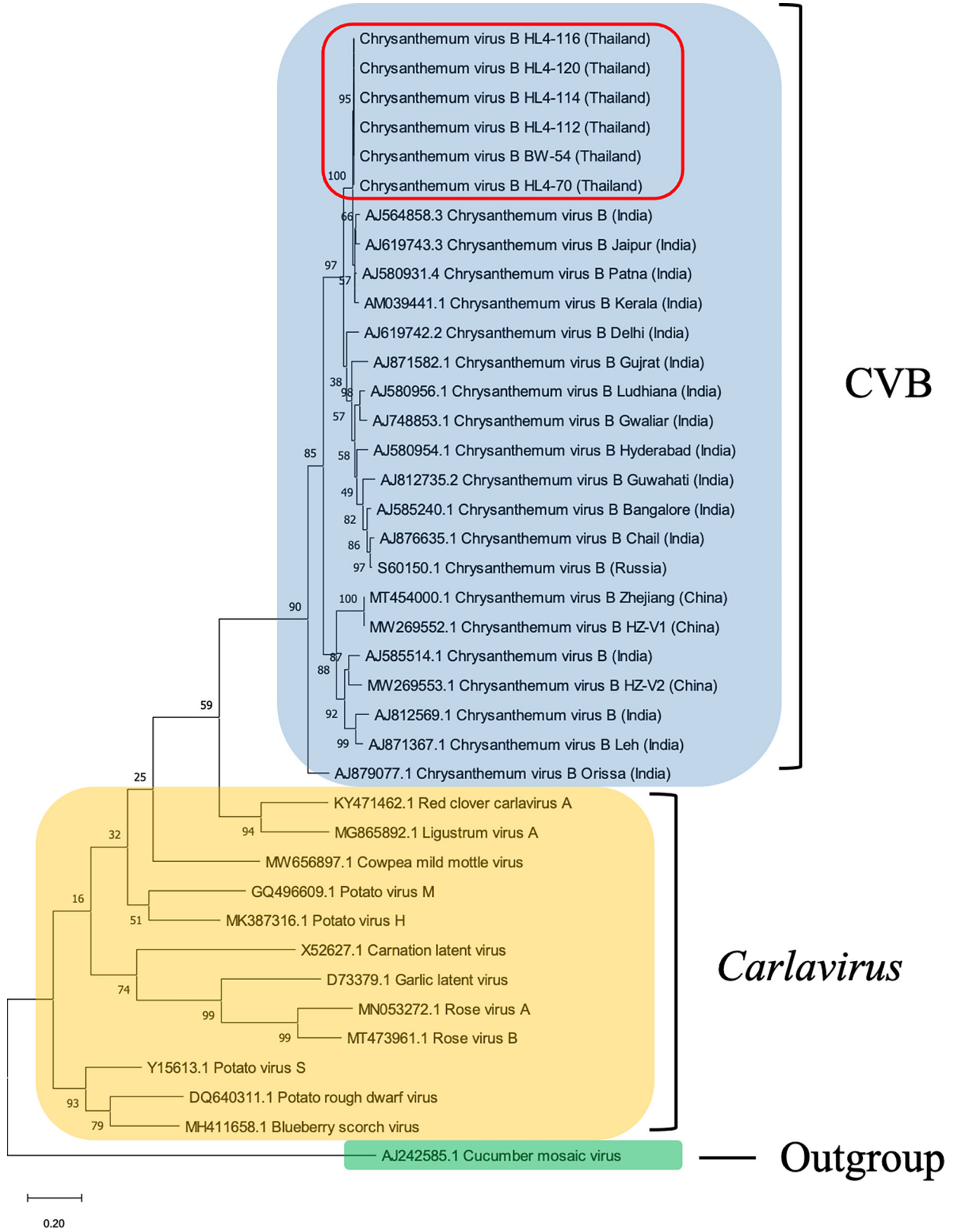

Figure 3. The phylogenetic relationship of CVB-BW-54 and CVB-HL4-70 (the red box) and members of the genus *Carlavirus* based on nucleotide sequences of the *CP* gene. *CMV* (accession №. AJ242585.1) was used as the outgroup. Multiple sequence alignments and construction of the phylogenetic tree were generated by ClustalW by the ML method with 1,000 replicates of bootstrap values performed by MEGA X program. CMV, cucumber mosaic virus; *CP*, coat protein; ML, maximum likelihood.

inoculated chrysanthemum at 30 dpi, with yellowing on the upper leaves. Two tobacco plants, *N. benthamiana* and *N. tabacum* cv. *Xanthi*, exhibited mosaic symptoms and malformation of newly developed young leaves at 7–10 dpi. Inoculated *P.* × *hybrida* showed systemic mosaic symptoms with malformation on the upper leaves at 21 dpi (Table 2).

LAMP primer design and optimisation of LAMP reaction

Four LAMP primers were designed: FIP, BIP, F3 and B3 (Table 1). The positions where LAMP primers anneal to the CVB genome, and the corresponding sequence, are illustrated in Figure 4. FIP and BIP primers were prepared at 16 µM stock concentration and F3 and B3 primers at 8 µM stock concentration.

The results show detection of a ladder pattern of LAMP products with incubation at 60 min for all tested temperatures (60 °C, 63 °C, 65 °C and 68 °C) (Figure 5A). Then, incubation times of 30 min, 45 min and 60 min for incubation at 65 °C were tested according to the manufacturer's recommendation. The results revealed that LAMP products were detected with incubation at 65 °C for 45 min and 60 min (Figure 5B). Based on the highest intensity of LAMP products, the optimal condition for CVB detection was incubation at 65 °C for 45 min. In all cases, the positive LAMP results consistently showed a colour change from pink to yellow, whereas the negative results consistently remained pink (Figures 5A and 5B).

Table 2. Symptoms induced by CVB on indicator plants after mechanical inoculation.

Indicator plants	Symptoms	
	Inoculated leaf	Upper leaf
Chenopodium amaranticolor	CS	–
C. quinoa	CS	–
Chrysanthemum × *morifolium*	–	Y
Nicotiana benthamiana	–	M, Ma
N. glutinosa	NS	–
N. tabacum cv. *Xanthi*	–	M, Ma
N. tabacum cv. *Samsun*	NS	–
Petunia × *hybrida*	–	M, Ma
Vigna uniculata	–	–

CS: chlorotic spot; CVB, chrysanthemum virus B; M, mosaic; Ma, malformation; NS, necrotic spot; Y, yellowing; –, no symptom.

Sensitivity assay

The LOD of LAMP for CVB plasmid was up to 100 fg (10^{-9}) (Figure 6A), which is 10^6 times higher compared to RT-PCR. The LOD of RT-PCR was up to 10^{-3} of diluted plasmid (1 ng) (Figure 6B). Colourimetric observation showed corresponding results with gel electrophoresis, whereby the colour of LAMP reactions of undiluted plasmid and plasmid diluted from 10^{-1} to 10^{-9} changed from pink to yellow, while the negative sample and plasmid diluted at 10^{-10} remained pink (Figure 6A).

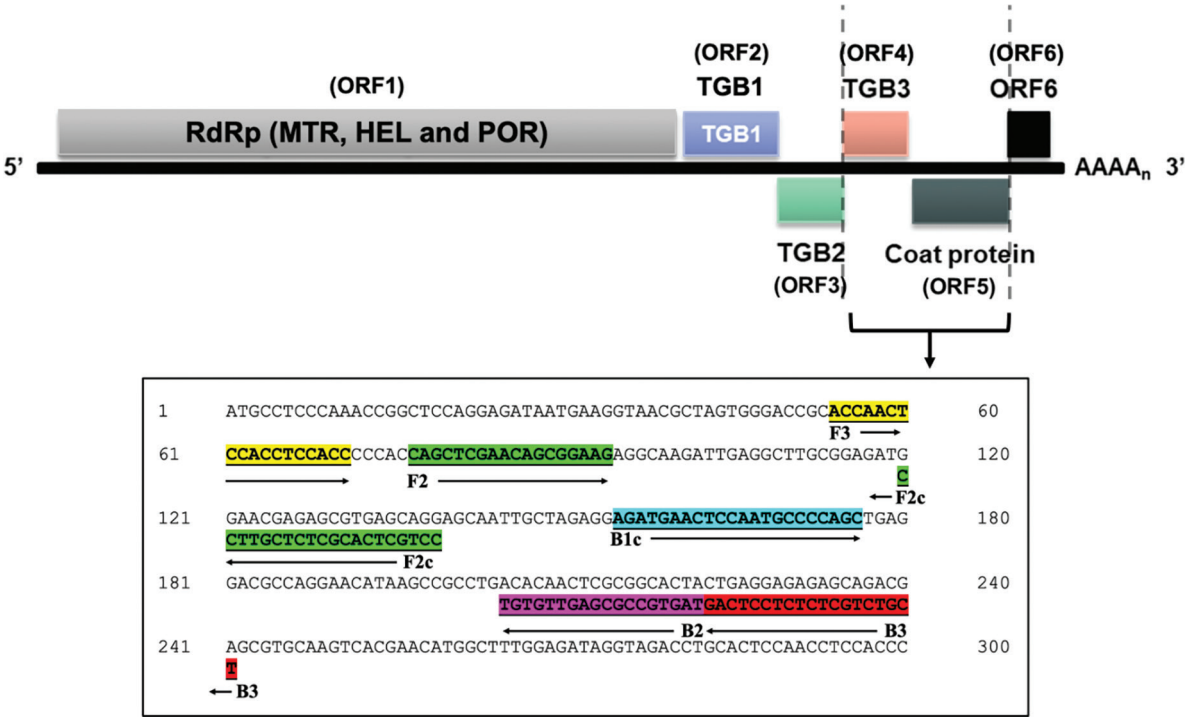

Figure 4. Diagram of LAMP primers' attachment on the partial ORF4 (*TGB3* gene) and ORF5 (*CP* gene) of CVB genome. All four LAMP primers including FIP (containing F2 and F2c), BIP (containing B2 and B1c), F3 and B3 were highlighted with different colours. BIP, backward inner primer; B3, backward primer; CVB, chrysanthemum virus B; FIP, forward inner primer; F3, forward primer; LAMP, loop-mediated isothermal amplification.

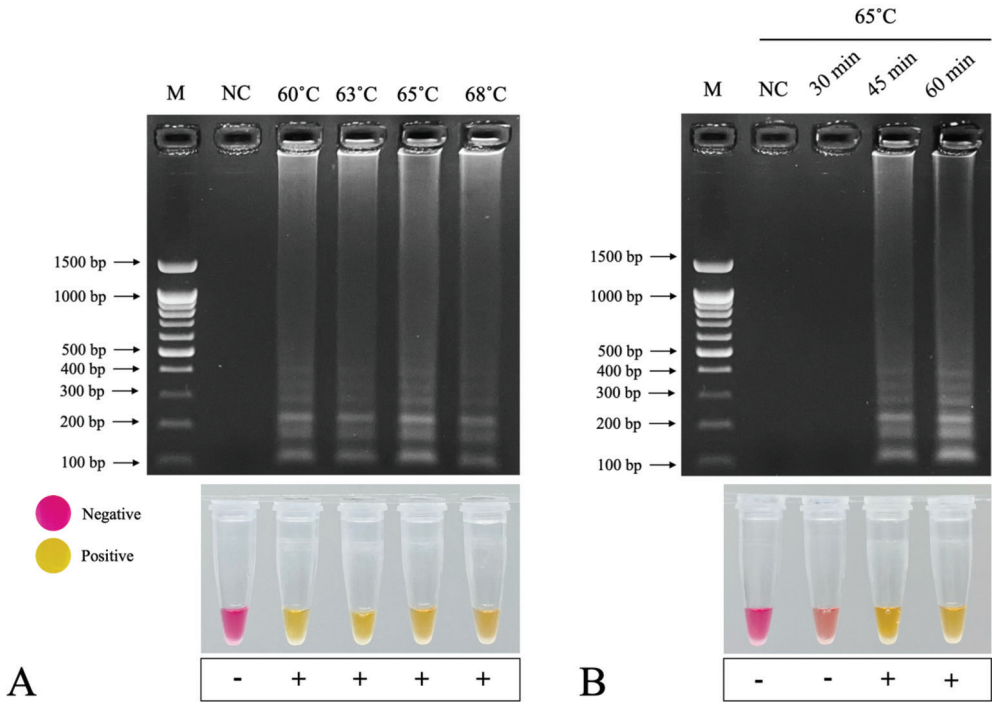

Figure 5. Optimisation of RT-LAMP for detection of CVB. (A) Optimisation of temperature at 60 °C, 63 °C, 65 °C and 68 °C for 60 min. (B) Optimising time of incubation at 65 °C for 30 min, 45 min and 60 min. CVB, chrysanthemum virus B; M, DNA ladder; NC, negative control (nuclease-free water).

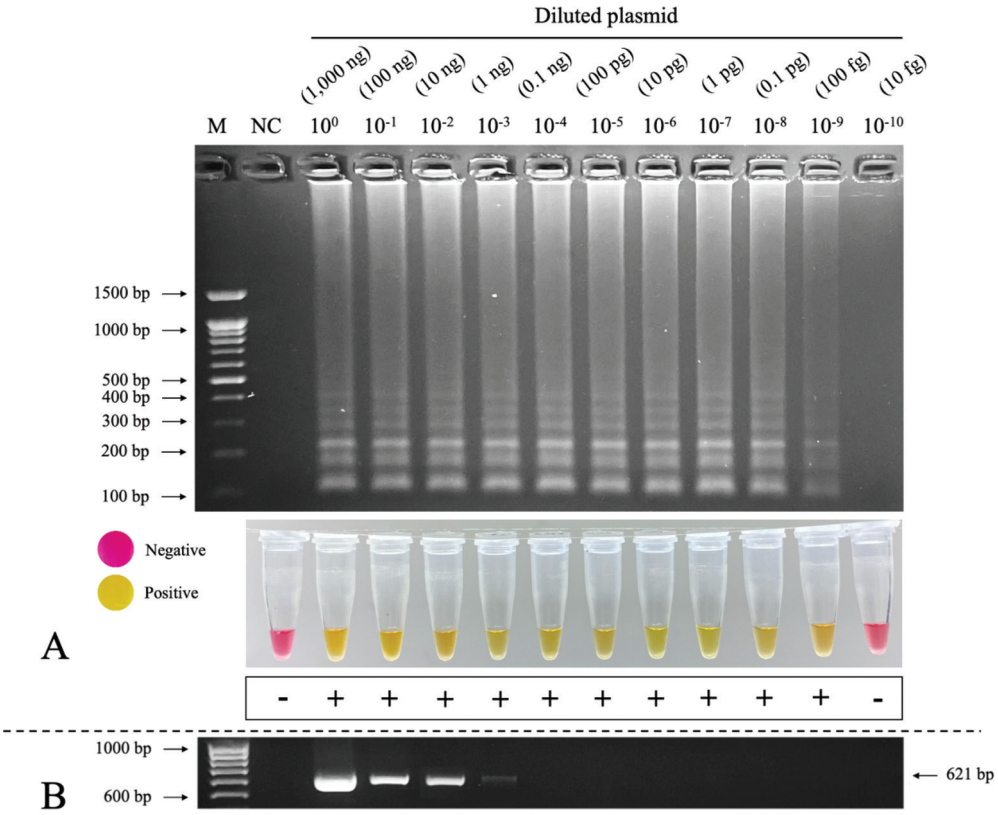

Figure 6. Sensitivity test of LAMP in the detection of CVB compared with RT-PCR detection. (A) LAMP-AGE (top) with the LOD at 10^{-9} diluted plasmid and colourimetric LAMP (bottom). (B) PCR with LOD at 10^{-3} diluted plasmid. AGE, agarose gel electrophoresis; CVB, chrysanthemum virus B; LAMP, loop-mediated isothermal amplification; LOD, limits of detection; M, DNA ladder; NC, negative control (nuclease-free water); PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction.

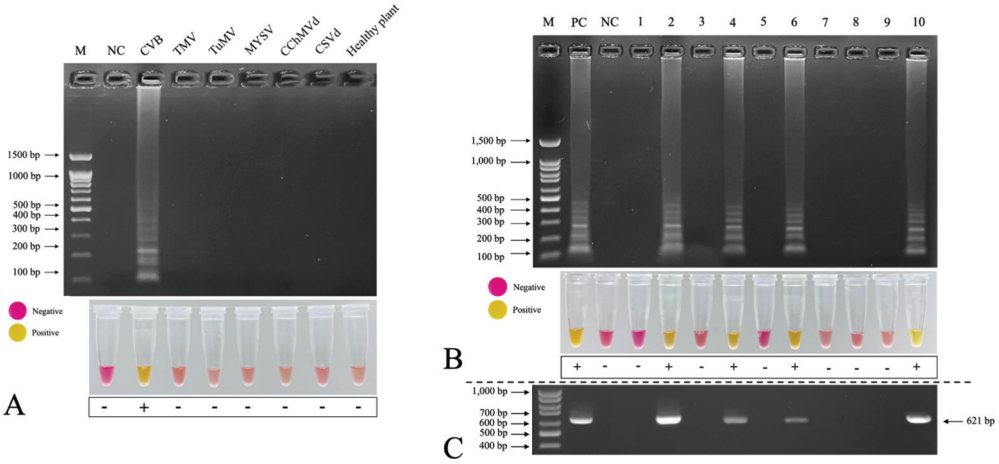

Figure 7. Specificity assay and evaluation of colourimetric RT-LAMP for detection of CVB. (A) The LAMP product was observed only in the lane of CVB. (B) Evaluation of colourimetric RT-LAMP for detection of CVB from random chrysanthemum samples. (C) RT-PCR detection. CChMVd, chrysanthemum chlorotic mottle viroid; CSVd, chrysanthemum stunt viroid; CVB, chrysanthemum virus B; LAMP, loop-mediated isothermal amplification; Lanes 1–10, chrysanthemum samples; M, DNA ladder; MYSV, melon yellow spot virus; NC, negative control (nuclease-free water); PC, positive control; RT-PCR, reverse transcription polymerase chain reaction; TMV, tobacco mosaic virus; TuMV, turnip mosaic virus.

Specificity assay and evaluation of colourimetric RT-LAMP performance

To assess cross-reactivity, LAMP reaction using the LAMP primers designed in this study was performed at the optimal condition (incubation at 65 °C for 45 min) with other viruses and viroids. The results show that the LAMP product was observed only in the CVB lane and not in other lanes corresponding to non-CVB viruses and viroids, with corresponding colourimetric results (Figure 7A).

Ten chrysanthemum samples were newly collected and used to test the efficacy of colourimetric RT-LAMP in CVB detection. The results showed that four samples were positive for CVB based on the observation of LAMP products analysed by gel electrophoresis and the colour change from pink to yellow of RT-LAMP reactions (Figure 7B, lanes 2, 4, 6 and 10). The negative results of RT-LAMP reactions remained pink (Figure 7B, lanes 1, 3, 5 and 7–9). RT-PCR was used to detect CVB compared to colourimetric RT-LAMP and showed the same results, detecting PCR products with 621 bp of CVB from four LAMP-positive samples (Figure 7C, lanes 2, 4, 6 and 10). According to the colourimetric evaluation of RT-LAMP results of CVB detection, in which 4 of 10 newly collected chrysanthemum samples were positive, the PDI of CVB in chrysanthemum of Thailand was recalculated as 5% (6/120 samples).

DISCUSSION

Chrysanthemum plants are susceptible to CVB infection; however, some cultivars can be tolerant to virus infection and remain symptom-free and unharmed, which does not have any effects on production and is referred to as latent infection (Yamamoto et al., 2001). On the other hand, susceptible chrysanthemum cultivars exhibit yellowing, mottling and vein banding symptoms (Hollings, 1957; Verma et al., 2003; Singh et al., 2012). In Taiwan, most CVB-infected chrysanthemum are symptom-free, with only a small percentage of plants exhibiting mild mottling symptoms on their leaves (Lin et al., 2005). Lin et al. (2005) reported that the symptoms are less likely to occur at lower temperatures; therefore, disease development in chrysanthemum depends on the cultivar and environmental conditions.

In India, CVB is generally found in coinfections with CMV and shows the characteristic symptoms (Verma et al., 2004). In this study, we assessed whether there was coinfection by attempting to detect CMV, TAV, CSNV, INSV, TSWV and ZYMV in chrysanthemum samples by RT-PCR, but the results were negative in all cases (data not shown). CChMVd and CSVd were detected, but not in samples that were positive for CVB (data not shown). Therefore, we assumed that the CVB infections in the chrysanthemum samples in this study represent a single infection.

As mechanical inoculation of CVB in chrysanthemum is difficult (Hollings, 1957), we concluded that *N. benthamiana, N. tabacum* cv. *Xanthi* and *P.* × *hybrida* are suitable models for assessing CVB propagation, because these indicator plants showed systemic symptoms similar to those reported by Martelik and Mokra (1998) and Singh et al. (2012) when inoculated in this study. The appearance of symptoms was not clearly observed on CVB-inoculated chrysanthemum. Indian CVB isolates have a narrow host range restricted to *N. clevelandii, N. glutinosa*, petunia and *Vicia faba* (Verma et al., 2003). The Thai CVB isolate had a relatively narrow host range restricted to chrysanthemum, a few *Nicotiana* species and petunia, although CVB had *V. faba* (Leguminosae family) and tetragonia (Aizoaceae

family) as systemic hosts (Megan et al., 2001; Verma et al., 2003). Furthermore, CVB pathogenicity in *N. benthamiana*, the experimental propagative plant, and chrysanthemum was confirmed by TEM.

RT-LAMP for detecting CVB was previously developed and reported by Liu et al. (2014); in that report, detection condition was optimal with incubation at 63 °C for 60 min, whereby LAMP demonstrated 10^3 times higher sensitivity than conventional PCR. In this study, CVB was successfully detected by colourimetric RT-LAMP with incubation at 65 °C for 45 min, whereby the sensitivity was 10^6 times higher compared to RT-PCR, and positive results were easily observed by the change of colour from pink to yellow. The duration of LAMP detection did not exceed 75 min, with gel electrophoresis included (30 min), being overall shorter than RT-PCR (2.0–2.5 h). Gel electrophoresis is not necessary for colourimetric LAMP because the colour change in LAMP reactions can be visualised by the naked eye. Therefore, colourimetric RT-LAMP is complete within 45 min. This indicates that the RT-LAMP method for detecting CVB developed in this study is more effective than the previous protocol based on its shorter procedure time, higher sensitivity and simple visualisation of results.

CONCLUSIONS

In conclusion, we report and describe CVB infection in chrysanthemum from Thailand, along with the molecular and biological characterisation of CVB isolate BW54 detected in this study. Colourimetric RT-LAMP was developed by designing new sets of LAMP primers, which demonstrated successful detection of CVB from chrysanthemum. Moreover, the time for colourimetric RT-LAMP detection is shorter than RT-PCR, reducing processing time by approximately 2 h. When using colourimetric observation, gel electrophoresis is not needed, which means colourimetric RT-LAMP detection can be achieved within 45 min. A comparison of nucleotide sequences of the *CP* gene of these isolates showed that they shared 95.15% identity with other CVB isolates from GenBank. Biological indexing showed that some species of tobacco and petunia could be used as propagative host plants. CVB was observed to have slightly flexuous rod-shaped particles under TEM. To our best knowledge, this is the first report of CVB detected from chrysanthemum in Thailand, along with descriptions of the molecular and biological characteristics and the development of the colourimetric RT-LAMP technique for improving detection efficiency. This colourimetric RT-LAMP technique has great potential for use in CVB detection, identification and the development and application of control strategies.

AUTHOR CONTRIBUTIONS

S.S. designed, performed the experiment and wrote the paper. T.M. and P.T. edited the paper. K.V. and K.K. provided chrysanthemums and locations for sampling and O.R. designed the experiment, conceptual advice and edited the paper.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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