

The development of a duplex RT-qPCR assay for the simultaneous detection of cycas necrotic stunt virus and lychnis mottle virus in *Paeonia lactiflora*

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Abstract

In this study, a duplex probe based reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay was utilized to simultaneously detect cycas necrotic stunt virus (CNSV) and lychnis mottle virus (LycMoV) in *Paeonia lactiflora* collected from various locations in South Korea. CNSV and LycMoV infections were verified by using conventional reverse transcription polymerase chain reaction (RT-PCR) using gene-specific primers. Due to peony's high secondary metabolites, multiple standard templates in the form of both DNA and RNA were evaluated. The circular plasmid was observed to produce the finest results and was used in dye-based qPCR to select the best-performing primers characterized by their ability to yield a low threshold cycle (Ct) and high fluorescence. The high precision quantification duplex probe-based qPCR assay was conducted and then optimized. The combination of primer concentration of 5 pmol/μl coupled with probe concentration of 4 pmol/μl at the annealing temperature of 57 °C produced stable and consistent amplification plots and standard curves. This combination demonstrated the capability to simultaneously detect plasmid DNA of both CNSV and LycMoV at concentrations as low as 10⁻⁶ ng/μl. These primer sets and optimum conditions were applied in RT-qPCR to detect total RNA of peony leaves co-infected with CNSV and LycMoV. Successful detection occurred with a slightly weaker sensitivity, having a detection limit of 10⁻³ ng/μl. The use of duplex probe-based RT-qPCR assay demonstrated in this study should improve the virus screening process of CNSV and LycMoV, leading to a reduction of the spread of these two plant viruses.

Introduction

Peony is a perennial plant native to Central Asia and Southern Europe and is cultivated for ornamental and medicinal purposes. *Paeonia lactiflora*, famously known as Chinese peony, is a cut flower characterized by its vibrant flowers which represent wealth and nobleness in China. Considered the king of flowers in the cut flower industry, it is also known for its medicinal properties in traditional East Asian medicine, most noticeably being anti-cancer (Cheng et al. 2011; Yu et al. 2007). A number of studies have been conducted on peonies in South Korea ranging from those on breeding, propagation, and micropropagation, as well as those on peony's medicinal properties (Ahn et al. 2018; Im et al. 2017; Lim et al. 2013; Lim et al. 2011; Yu 1970). Recent studies have shown that *P. lactiflora* is susceptible to viruses such as CNSV, LycMoV, tobacco rattle virus (TRV), gentian kobu-sho associated virus (GKaV), and amazon lily mild mottle virus (ALiMMV), which could affect its production (Robertson et al. 2009; Shaffer et al. 2019; Shaffer, Michener, Vlasava, Chotkowski, et al. 2022; Shaffer, Vakić, & Tzanetakis 2021; Uehara-Ichiki et al. 2020; Yoon et al. 2024).

CNSV (*Nepovirus*) infections showed symptoms of dwarfing and twisting on young leaves and leaving behind chlorotic or necrotic spots on mature leaves of *Cycas revoluta* (Kusunoki et al. 1986). CNSV is known to infect alfalfa (Jiang et al. 2019), Easter lily (Wylie et al. 2012), and peony (Ochoa-Corona 2003; Shaffer et al. 2019; Tang et al. 2020). In South Korea, CNSV was found to infect numerous other plants, including *Cnidium officinale* (Igori et al. 2020), *Daphne odora*, and *Paeonia suffruticosa* (Lim et al. 2019). In this study, *P. lactiflora* was confirmed to be susceptible to CNSV (Yoon et al. 2024). The infections are

generally asymptomatic in peony cultivars. The vector responsible for spreading CNSV has not been identified yet, but the like of *Nepovirus* is well known for its capability to transmit via seed, pollens, sap inoculation, and nematodes (Shaffer et al. 2019). LycMoV, *Stralarivirus*, is in the same *Secoviridae* family as CNSV, and its genome organization was thoroughly identified (Yoo et al. 2015). LycMoV is known to infect *Lychnis cognata* (Yoo et al. 2015), *Rehmannia glutinosa* (Uehara-Ichiki et al. 2016), *Vincetoxicum acuminatum* (Fujimoto et al. 2018), *Paeonia lactiflora* (Shaffer et al. 2019), *Angelica sinensis*, *Medicago sativa*, and *Prunus persica* (Jin, Zhang, Su, Xie, et al. 2023). *A. sinensis* infected with LycMoV showed symptoms of leaf mosaic, chlorosis, yellow blotches, and mottling (Jin, Zhang, Su, Xie, et al. 2023). LycMoV can be mechanically transmitted to *Catharanthus roseus* (Periwinkle), resulting in symptoms, such as chlorosis, stunting, rosette, and leaf curl (Shaffer et al. 2019). Other means of LycMoV transmission include nematode transmission and transmission via propagation materials (Shaffer, Michener, et al. 2021).

The presence of both CNSV and LycMoV in *P. lactiflora* has been reported in North America through high-throughput sequencing (HTS) analysis, and verified by using conventional RT-PCR. Shaffer et al. (2019) reported all peonies infected with LycMoV to be co-infected with CNSV. Jia et al. (2021) performed HTS analysis of small RNA and mRNA of tree peonies exhibiting symptoms of yellowing, leaf rolling, stunted growth, and decline. CNSV and LycMoV were identified in mixed viral infections with other viruses such as apple stem grooving virus (ASGV), grapevine line pattern virus (GLPV), and three new viruses. The population genetics of both CNSV and LycMoV were thoroughly studied through HTS analysis (Shaffer, Michener, et al. 2021; Shaffer, Michener, Vlasava, Chotkowski, & Tzanetakis 2022). Shaffer, Michener, Vlasava, Chotkowski and Tzanetakis (2022) analyzed CNSV population genetics in an attempt to better understand virus epidemiology to improve diagnostic accuracy, and developed a multiplex RT-PCR assay to detect all published isolates of CNSV. Shaffer, Michener, et al. (2021) hypothesized that the introduction of LycMoV and other plant viruses to North America may be due to peony imports from Asia, and that the spread of LycMoV in the United States was due to propagation practices rather than vector-mediated transmission. Jin, Zhang, Su, Wang, et al. (2023) established a detection system for LycMoV in *Angelica sinensis* by using a combination of a rapid colloidal gold immunochromatographic assay (GICA) with a reverse transcription loop mediated isothermal amplification assay (RT-LAMP), resulting in an assay with the sensitivity of 2.53×10^{-3} ng/ μ l.

qPCR, being a fluorescence-based PCR, can be used as a highly sensitive virus screening tool capable of distinguishing the presence of multiple viruses. Due to qPCR's superior quicker detection of often smaller concentrations of the target gene, coupled with high specificity of hydrolysis probe, probe-based qPCR can be utilized to simultaneously detect plant viruses. To fulfill the lack of a screening method for simultaneous detection of both viruses, this study demonstrated the application of a duplex hydrolysis probe-based RT-qPCR using the high precision quantification method for the detection of CNSV and LycMoV in *P. lactiflora*. The study began from (1) detecting viruses by using conventional RT-PCR which were then confirmed through sequencing data, (2) using the dye-based qPCR for primer selection, (3) conducting standard curve comparisons between DNA and RNA standard templates, (4) optimizing the probe-based qPCR, and, finally, (5) establishing the duplex probe-based RT-qPCR.

Materials and methods

Plant materials and sample preparations

The symptomatic peony leaves were collected from fields and kept in plastic bags. They were refrigerated at 4 °C upon arrival in the laboratory, which was usually less than 6 hours after collection. The total RNA was extracted from 50 mg of symptomatic leaves by using a filter-based spin basket method via the Plant RNA mini kit (Onsol, South Korea), following the manufacturer's instructions with slight modifications as follows. The leaf was ground immersed in liquid nitrogen using mortar and pestle, it was then transferred to a 2-ml centrifuge tube before the necessary buffer was added. RNA purity and quantity were acquired by using Epoch Microplate Spectrophotometer (BioTek, United States).

The symptomatic samples were screened for peony-infecting viruses, such as CNSV, LycMoV, TRV, ALiMMV, GKAV, citrus leaf blotch virus (CLBV), and cucumber mosaic virus (CMV) via conventional RT-PCR using the gene-specific primers derived from the published studies (Cardin et al. 2010; Gress et al. 2017; Robertson et al. 2009; Shaffer, Michener, Vlasava, Chotkowski, et al. 2022; Shaffer, Vakić, & Tzanetakis 2021). The RT-PCR reaction was conducted by using the TOPscript RT-PCR kit (Enzynomics, South Korea). The components consisted of 5 µl of TOPscript One-step RT-PCR kit, 1 µl of forward primer (10 pmol/µl), 1 µl of reverse primer (10 pmol/µl), and 1 µl of RNA in a final reaction volume of 20 µl. The mixture was heated at 50 °C for 30 minutes to construct the cDNA library, which was followed by an initial denaturing stage at 95 °C for 10 minutes and 40 cycles of denaturing at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, and elongation at 72 °C for 40 seconds, then finished with a final elongation stage at 72 °C for 5 minutes. Gel visualizations were conducted by using the Gel Doc XR+ (Bio-Rad, United States) in 2% agarose gels electrophorized at 100 V for 35 minutes. All of the isolated RNAs were then stored in -70 °C freezer.

Primer and probe design

The sequencing data were used to aid in primer and probe design. The CNSV and LycMoV PCR products were ligated to pGEM-T Easy Vector (Promega, United States), following the manufacturer's guidelines. The competent cell of choice for the transformation was the DH5α chemically competent *E. coli* (Enzynomics, South Korea). Plasmid DNA purification was conducted by using the EZ-Pure Plasmid Prep Kit. Ver. 2 (Enzynomics, South Korea). The plasmid DNAs were then sent to the BIONEER company, one of South Korean sequencing service provider located in Daejeon.

Multiple sequence alignments were conducted by using the clustal omega tool provided by EMBL-EBI website (<https://www.ebi.ac.uk/jdispatcher/msa/clustalo>). qPCR primers and probes were selected in the conserved region of CNSV coat protein and LycMoV protease cofactor with the help of PrimerQuest tool provided free of use in Integrated DNA Technologies website (<https://idtdna.com/PrimerQuest/Home/Index>) and the sequencing data from BIONEER. The CNSV qPCR primers and probe were selected from the multiple sequence alignment of LC785683, MW328854, MW328868, MW328858, LC577869, and JN127337. The LycMoV qPCR primers and probe were selected

from the multiple sequence alignment of LC785521, MN259155, MH878973, MH878974, MH878970, and MH878971 (Table 1 & Fig S1).

Table 1

The RT-PCR and qPCR primers and probes used in the study to detect cycas necrotic stunt virus (CNSV) and lychnis mottle virus (LycMoV)

Target viruses	Types of PCRs	Sequences 5' → 3'	Primer size (mer)	Amplicon size (bp)
CNSV	RT-PCR	F: CACCAAGTCCAGTTCTCTCTTATC	24	336
		R: CCACTCCTGTATCAGTATCAAC	22	
	qPCR	F: CAAAGTGTGGGAGAGGAGCTAC	22	102
		R: GGGCAAGAACAAGTCCAAATC	21	
		Probe: FAM-ACCAAGTCCAGTTCTCTCGTATCCCT-BHQ1	26	
LycMoV	RT-PCR	F: GGAGTCATGGCAAAGCTACG	20	412
		R: CAAGCACCTCAATTATTTGCTCATC	25	
	qPCR	F: TGC GTGCGTTACTTCAACC	19	112
		R: AGGATAGCCAGAGGAATACCC	21	
		Probe: Cy5-TGCTCCACAATTGCCTTCAATCCC-BHQ3	24	

Primer selection by using the dye-based qPCR

The dye-based qPCR was performed to select the best-performing primers, which produced a low Ct value and high fluorescence, for probe-based detection. The reactions were conducted by using SYBR Green double-stranded DNA-binding dye via the TOPreal SYBR Green qPCR High-ROX PreMIX (Enzynomics, South Korea) on the plasmid DNA of the corresponding target viruses. The components consisted of 10 µl of TOPreal qPCR 2X PreMIX (SYBR Green with high ROX), 1 µl of forward primer (10 pmol/µl), 1 µl of reverse primer (10 pmol/µl), and 1 µl of DNA in a final reaction volume of 20 µl. The mixture underwent the initial denaturing stage at 95 °C for 10 minutes and 40 cycles of denaturing at 95 °C for 10 seconds, annealing at 55 °C for 15 seconds, and extension at 72 °C for 30 seconds. The melt curve analysis was conducted by heating the mixture to 65 °C for 10 seconds and gradually increased to 95 °C by 0.5 °C/s. These qPCR experiments were conducted in the CFX Opus 96 Real-Time PCR systems (Bio-Rad, United States).

Comparisons of the standard curves by using the dye-based qPCR

Both the DNA and RNA standard curves were constructed and evaluated for high precision quantification using qPCR and RT-qPCR, respectively (Table 2). The evaluation was conducted using CNSV as the viral

target of interest. All of the nucleic acids were quantified twice using the Epoch Microplate Spectrophotometer (BioTek, United States), averaged, and diluted with nuclease free water to 1 ng/μl; and then serially diluted to 10⁻⁷ ng/μl. The dye-based qPCR with SYBR Green dsDNA-binding dye was used for the evaluation. These experiments were conducted in duplicates using the StepOne Real-Time PCR system (Applied Biosystems, United States).

Table 2

Comparison of standard curves to select the best template for the high precision quantification dye-based qPCR using the standard curve experiments.

	Types of templates			
	DNA template		RNA template	
	Circular plasmid	Linearized plasmid	Total RNA	Transcript RNA
Types of qPCRs	qPCR	qPCR	RT-qPCR	RT-qPCR
Kits	TOPreal SYBR Green qPCR High-ROX PreMIX	TOPreal SYBR Green qPCR High-ROX PreMIX	TOPreal SYBR Green RT-qPCR High-ROX Kit	TOPreal SYBR Green RT-qPCR High-ROX Kit
Method used to produce the templates	pGEM-T Easy Vector	pGEM-T Easy Vector linearized by using restriction enzyme	Filter-based spin basket isolation (total RNA isolation)	MEGAscript® T7 Transcription Kit (in vitro transcription)
These experiments were conducted in duplicates by using the StepOne Real-Time PCR system (Applied Biosystems, United States).				

Circular plasmid DNA and linearized plasmid DNA were used for the DNA standard curves. Circular plasmid DNA was linearized by using a restriction enzyme carefully selected from the vector map of pGEM-T Easy Vector with the aid of the sequencing data of the said amplicon to avoid cleaving the target of interest. Total RNA and transcript RNA were used for the RNA standard curves. The total RNA was obtained by using filter-based spin basket method. The transcript RNA was synthesized via in vitro transcription using the MEGAscript T7 Transcription kit (Thermo Fisher, United States).

Optimizations of the probe-based qPCR

The probe-based qPCR was performed to assess the primer and probe specificity among CNSV and LycMoV by using the TOPreal Multi-Probe qPCR PreMIX (Enzynomics, South Korea). The components consisted of 10 μl of TOPreal qPCR 2X PreMIX (TaqMan Probe for multiplex), 1 μl of probe (4 pmol/μl), 1 μl of forward primer (10 pmol/μl), 1 μl of reverse primer (10 pmol/μl), 1 μl of DNA in a final reaction volume of 20 μl. The mixture underwent the initial denaturing stage at 95 °C for 10 minutes and 40 cycles of denaturing at 95 °C for 10 seconds and annealing and extension at 55 °C for 30 seconds.

The parameters for reaction optimization included primer and probe concentrations, annealing temperatures, and detection limit. The optimum condition was decided based on the stability of the amplification plot, low Ct value, and similarly high relative fluorescence units (RFU). The RFU became a factor in the optimizations due to the duplex experiment requiring similar fluorescence levels from both targets. The primer and probe concentrations included 10 pmol/μl, 5 pmol/μl, and 1 pmol/μl primers combined with 4 pmol/μl, 2 pmol/μl, and 1 pmol/μl probes. The best combination was then selected for the next optimization stage. The annealing temperature tested were 63 °C, 61 °C, 59 °C, 57 °C, 55 °C, and 53 °C. The best combination was selected for the detection limit test using the plasmid DNA with the concentrations ranging from 1 ng/μl to 10⁻⁷ ng/μl. These qPCR experiments were conducted in the CFX Opus 96 Real-Time PCR systems (Bio-Rad, United States).

Application of the duplex probe-based RT-qPCR

The duplex RT-qPCR reaction was conducted by using the TOPreal Multi-Probe RT-qPCR kit (Enzynomics, South Korea). The primer concentration, probe concentration, and annealing temperature were obtained from the duplex probe-based qPCR optimization experiments. The reaction components consisted of 5 μl of TOPreal One-step RT qPCR kit (TaqMan Probe for multiplex), 1 μl of each probe (4 pmol/μl), 1 μl of each forward primer (5 pmol/μl), 1 μl of each reverse primer (5 pmol/μl), 1 μl of DNA in a final reaction volume of 20 μl. The mixture underwent the reverse transcription stage at 50 °C for 30 minutes, the initial denaturing stage at 95 °C for 10 minutes, and 40 cycles of denaturing at 95 °C for 5 seconds and annealing and extension at 57 °C for 30 seconds. These qPCR experiments were conducted in the CFX Opus 96 Real-Time PCR systems (Bio-Rad, United States).

Results

Virus detection by using RT-PCR and verification through the sequencing data

The leaf samples infected with CNSV showed symptoms of mottle and chlorotic spots (Fig. 2a), while the leaf co-infected with CNSV and LycMoV showed chlorotic spots and wrinkles which expanded from the leaf base to the leaf end (Fig. 2b). The presence of both CNSV and LycMoV were confirmed through RT-PCR resulting in amplicon size of 336 bp for CNSV and 412 bp for LycMoV (Fig. 1c). The entire coat protein (CP) region of CNSV and the 412 bp-region of LycMoV were cloned, sequenced, and deposited in the NCBI library as LC785683 and LC785521, respectively. BLAST analyses of the sequences confirmed the presence of CNSV and LycMoV. Our CNSV CP isolate shared a 91.54% similarity to another CNSV isolate from peonies in the United States (MW328854), a 91.26% identity with another CNSV isolate from Korean peony (LC577869), a 90.42% similarity with another CNSV isolate from lily (JN127337), and a 90.43% similarity with CNSV reference sequence derived from cycas (NC_003792). Our LycMoV isolate an 86.34% similarity with another secovirus isolate from peony in China (MN259155) and a 93% similarity with another LycMoV isolate from peony in the United States (MH878973).

Primer selection by using the dye-based qPCR

The CNSV primer set was selected from 26 CNSV primer sets targeting different regions of the CNSV genome. The LycMoV primer set was selected from 6 LyMoV primer sets targeting the Pro-C region of the LycMoV genome. The best primer sets were selected based on having a low Ct value, high fluorescence, and consistent standard curves and amplification plots. The primer specificity was assessed by performing dye-based qPCR using nucleic acids from other target viruses, which included CNSV, LycMoV, TRV, and GKaV. The CNSV and LycMoV primer sets were specific to their own target virus. Neither the amplification peak nor the Ct value was produced from the non-target DNA. The melt curve assay showed a sole peak, indicating the specificity of both primer sets. Negative controls being ultra pure water were undetectable in all cases, reinforcing the certainty that all of the reactions were performed well.

Comparisons of the standard curves by using the dye-based qPCR

The amplification data for each template was shown in Table 3. Experiments were conducted in duplicates, resulting in a mean Ct value. At the highest concentration of 1 ng/μl, the DNA templates showed the lowest Ct value compared to RNA templates, in which the lowest C_t value was from the linearized plasmid at 4.15. At the lowest concentration of 10^{-7} ng/μl, the DNA templates were still detectable, whereas the RNA templates could no longer be determined. Although the linearized plasmid had lower Ct value at the highest concentration compared to the circular plasmid, as the concentration kept getting lower, specifically after 3 times of 10-fold serial dilution at 10^{-3} ng/μl, the Ct value of both templates matched for the rest of the dilutions. For the RNA templates, the detectable range was 10^{-3} ng/μl because the Ct values of RNA templates after 10^{-3} ng/μl were either inconsistent or undetermined, indicating that the fluorescence signals were unstable and unreliable. This inaccuracy was observed through the standard curve in which the R^2 value of the total RNA template was only 0.781 while the transcript RNA template was 0.722, both of which were not close to 1, enforcing the claim that the detection limit of the RNA templates were 10^{-3} ng/μl (Fig. 1e & g). For the DNA templates, on the other hand, the R^2 value of the circular plasmid was 0.992 and the linearized plasmid was 0.995, both of which were close to 1, indicating a very high accuracy (Fig. 1a & c). Based on the amplification plots, DNA templates produced equally spaced plots from the early stage of the amplification cycles, which were more desirable than amplification plots produced by RNA templates which were at the later stage of the amplification cycles. Due to the similar performance of the circular plasmid and the linearized plasmid, the circular plasmid was the template of choice for the remainder of the experiment, which included both the dye-based and probe-based qPCR experiments.

Table 3

The amplification data (Ct value) of the different types of templates produced within the range of 1 ng/μl to 10⁻⁷ ng/μl

Quantity (ng/μl)	Mean Ct value			
	DNA template		RNA template	
	Circular plasmid	Linearized plasmid	Total RNA	Transcript RNA
1	7.371285	4.152771	21.7316	22.78104
10 ⁻¹	10.98652	8.159449	27.08916	26.67378
10 ⁻²	14.67084	12.9701	31.85391	33.13015
10 ⁻³	17.68555	17.55114	35.84479	35.323
10 ⁻⁴	22.49335	21.65792	34.83543	Undetermined
10 ⁻⁵	26.85077	26.30221	35.86761	34.73265
10 ⁻⁶	30.54506	32.54072	34.80863	Undetermined
10 ⁻⁷	37.11721	37.75296	Undetermined	Undetermined
The DNA templates were circular plasmid and linearized plasmid. The RNA templates were total RNA and transcript RNA. The target of interest was cycas necrotic stunt virus (CNSV) being amplified by using CNSV qPCR primer (102 bp). The primer concentration was 10 pmol/μl, and the annealing temperature was 55 °C. These experiments were conducted in duplicates by using the StepOne Real-Time PCR system (Applied Biosystems, United States).				

Optimizations for the probe-based qPCR

The selected primers were compatible with their hydrolysis probes, and their specificities corresponded to their targets (Fig S2). Primer concentration of 5 pmol/μl, probe concentration of 4 pmol/μl, and annealing temperature of 57 °C were found to produce the best results, as well as decent RFU (Table S2–4). The templates for the standard curve construction were artificially made plasmid DNA mixture with the range from 1 ng/μl to 10⁻⁷ ng/μl. The R² value for the CNSV slope was 0.998, while the LycMoV slope was 0.993, indicating a high accuracy (Fig. 4a). The detection limit was 10⁻⁶ ng/μl for both viruses. The negative control was undetectable for both sets of primers. The selected primers from the dye-based qPCR functioned properly, while the Ct values were almost identical. When the RFU values from the dye-based qPCR (Fig. 1b & d) and from the probe-based qPCR (Fig. 4b & c) were compared, there was a noticeable drop for both targets.

The application of the duplex probe-based RT-qPCR

The MIQE checklist is provided in Supplementary Table 1 (Table S1) (Bustin et al. 2009). The optimum conditions of a primer concentration at 5 pmol/μl, a probe concentration at 4 pmol/μl, and an annealing

temperature at 57 °C were applied to detect CNSV and LycMoV in the form of total RNA by using the duplex probe-based RT-qPCR. While the detection was possible, the assay was slightly less sensitive than qPCR (Fig. 3). The R^2 value of the CNSV slope was 0.985 and the LycMoV slope was 1, graphed as a 4-magnitude standard curve. The detection limit was 10^{-3} ng/μl for both viruses. The qPCR efficiencies were 91.5% for CNSV primer and 91.7% for LycMoV (Fig. 3a). The negative control was undetectable for both sets of primers.

Discussions

The application of qPCR technology in the field of plant disease diagnostic results in a big leap of possibilities thanks to its powerful and sensitive diagnostic capabilities with increased accuracy and scalability that can be adopted into many biomolecular experiments and is shown to be superior to many current and previously adopted techniques. Nonetheless, there remain distinct parameters that can heavily influence the results produced from qPCR experiments. Experiments performed on the same sample can, in the worst cases, result in statistically different values of target quantity or copy number (Smith et al. 2006).

While a number of methods have been developed for the detection of either CNSV or LycMoV in peonies (Jia et al. 2021; Shaffer et al. 2019; Shaffer, Michener, et al. 2021; Shaffer, Michener, Vlasava, Chotkowski, & Tzanetakis 2022). However, a detection method taking advantage of the high sensitivity and specificity of qPCR has not been established yet until this study. The sensitivity of this optimized duplex RT-qPCR assay was comparable to a study that used GICA-RT-LAMP to detect LycMoV in *A. sinensis* (Jin, Zhang, Su, Wang, et al. 2023). This assay can be improved through additionally purifying the standard templates resulting in a more accurate Ct value. Due to the need of standard templates quantification via a separate instrument, for example spectrophotometric measurements or fluorometric measurements, measurement of samples with low impurity would lead to incorrect quantity of target nucleic acids. Beinhauerova et al. (2020) used a digital PCR (dPCR) in replacement of a spectrophotometer to independently quantify stock standards used in the qPCR experiments. A droplet dPCR (ddPCR) was found to be able to quantify the diluted concentration of a stock standard with only 10% variation from the expected value, compared to spectrophotometric measurements which varied up to 98%. This finding can revolutionize nucleic acid quantification and unify the interpretation of qPCR data, making it possible to make comparisons across laboratories and allow the qPCR results to speak the “same language.”

The circular plasmid was used as the standard template for the dye-based and probe-based qPCR. The circular plasmid, consisting of the vector and the insert, was enclosed and often time supercoiled which made it more stable than the linear plasmid or open circular plasmid. This complex formation was more difficult to denature which impacts the first few PCR cycles, suppresses the amplification efficiency, and alters the Ct value to be higher by 2.65 to 4.38 times when compared to linear plasmid (Chen et al. 2007; Hou et al. 2010; Shirima et al. 2017). Similar results were observed in this study as the Ct value produced from circular plasmid at 1 ng/μl was 7.37, whereas the Ct value produced by the linearized plasmid at the

same concentration was only 4.15 (Table 3). As the concentrations of the plasmids became more diluted at about 10^{-3} ng/μl, the Ct value of both type of plasmids were almost identical with the circular plasmid at 17.68, whereas the linearized plasmid was 17.55 (Table 3). At the start of the exponential phase of PCR (16th to 25th cycle), where the cycle threshold is commonly positioned, the produced PCR products overcome the initial template concentration meaning that the effect of supercoiled strands was negligible. The circular plasmid became more favorable also due to the fact that the linearized plasmid required an additional step of cleavage by using restriction enzyme which may have introduced contaminants that could have impacts on the sensitivity of the final products. At the lowest concentrations of 10^{-6} ng/μl and 10^{-7} ng/μl, the Ct value of the circular plasmid was lower than the linearized plasmid indicating a slightly better sensitivity (Table 3). Due to their similarity and identical consistency, the circular plasmid was chosen to be used as the template of choice for the remainder of the experiment.

Conclusions

The aim to develop a duplex probe-based RT-qPCR to simultaneously detect CNSV and LycMoV in *P. lactiflora* in this study was achieved. This assay utilized high-precision quantification using standard curve produced by a series of diluted total RNA. The dye-based qPCR was used for primer selection. The probe-based qPCR was used for the assay optimization, resulting in the use of 5 pmol/μl primer and 4 pmol/μl probe for both viral primer sets coupled with an annealing temperature of 57 °C. This is the first study which successfully utilized RT-qPCR as a screening method for two peony viruses, being CNSV and LycMoV, with the detection limit of 10^{-3} ng/μl.

Declarations

Authors' contributions

Conceptualization, HJ; Methodology, HJ, CS; Formal analysis, CS; Investigation, CS, CK; Resources, HJ, JY; Writing-Original Draft Preparation, CS; Writing-Review and Editing, CS, HY; Supervision, HJ, JY; Funding Acquisition, HJ. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

References

1. Ahn, MS, Park, PH, Kwon, YN, Mekapogu, M, Kim, SW, Jie, EY, Jeong, JA, Park, JT, Kwon, OK (2018) Discrimination of Floral Scents and Metabolites in Cut Flowers of Peony (*Paeonia lactiflora* Pall.) Cultivars. Korean Journal of Plant Resources 31(6):641-651.
<https://doi.org/10.7732/kjpr.2018.31.6.641>
2. Beinhauerova, M, Babak, V, Bertasi, B, Boniotti, MB, Kralik, P (2020) Utilization of Digital PCR in Quantity Verification of Plasmid Standards Used in Quantitative PCR [Original Research]. Frontiers in Molecular Biosciences 7. <https://doi.org/10.3389/fmolb.2020.00155>
3. Bustin, SA, Benes, V, Garson, JA, Hellemans, J, Huggett, J, Kubista, M, Mueller, R, Nolan, T, Pfaffl, MW, Shipley, GL, Vandesompele, J, Wittwer, CT (2009) The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry 55(4):611-622.
<https://doi.org/10.1373/clinchem.2008.112797>
4. Cardin, L, Onesto, JP, Moury, B (2010) First Report of Cucumber mosaic virus in *Paeonia lactiflora* in France. Plant disease 94(6):790-790. <https://doi.org/10.1094/pdis-94-6-0790c>
5. Chen, J, Kadlubar, FF, Chen, JZ (2007) DNA supercoiling suppresses real-time PCR: a new approach to the quantification of mitochondrial DNA damage and repair. Nucleic Acids Research 35(4):1377-1388. <https://doi.org/10.1093/nar/gkm010>
6. Cheng, Y, Kim, CH, Shin, DI, Kim, SM, Koo, HM, Park, YJ (2011) Development of simple sequence repeat (SSR) markers to study diversity in the herbaceous peony (*Paeonia lactiflora*). Journal of Medicinal Plants Research 5(31):6744-6751. <https://doi.org/10.5897/Jmpr11.889>
7. Fujimoto, Y, Nijo, T, Hosoe, N, Watanabe, K, Maejima, K, Yamaji, Y, Namba, S (2018) Complete Genome Sequence of Lychnis Mottle Virus Isolated in Japan. Genome Announcements 6(25):10.1128/genomea.00535-00518.
<https://doi.org/doi:10.1128/genomea.00535-18>
8. Gress, JC, Smith, S, Tzanetakis, IE (2017) First Report of Citrus leaf blotch virus in Peony in the U.S.A. Plant disease 101(4):637-637. <https://doi.org/10.1094/pdis-08-16-1218-pdn>
9. Hou, Y, Zhang, H, Miranda, L, Lin, S (2010) Serious Overestimation in Quantitative PCR by Circular (Supercoiled) Plasmid Standard: Microalgal pcna as the Model Gene. PLOS ONE 5(3):e9545.
<https://doi.org/10.1371/journal.pone.0009545>
10. Igori, D, Lee, H-K, Yang, H-J, Lee, D-S, Kim, S-Y, Kwon, B, Oh, J, Kim, T-D, An, C, Moon, J-S, Lee, S-H (2020) First Report of the Cycas Necrotic Stunt Virus Infecting *Cnidium officinale* in South Korea. Plant disease 104(12):3275-3275. <https://doi.org/10.1094/pdis-01-20-0092-pdn>
11. Im, I-b, Im, B-H, Park, J-H, Im, M-H, Jang, J-H, Lee, I-Y (2017) Weed occurrence in peony (*Paeonia lactiflora*) fields. Weed & Turfgrass Science 6(3):165-178.
<https://doi.org/10.5660/WTS.2017.6.3.165>
12. Jia, A, Yan, C, Yin, H, Sun, R, Xia, F, Gao, L, Zhang, Y, Li, Y (2021) Small RNA and Transcriptome Sequencing of a Symptomatic Peony Plant Reveals Mixed Infections with Novel Viruses. Plant

- disease 105(12):3816-3828. <https://doi.org/10.1094/pdis-01-21-0007-re>
13. Jiang, P, Shao, J, Nemchinov, LG (2019) Identification of the Coding-Complete Genome of Cycas Necrotic Stunt Virus in Transcriptomic Data Sets of Alfalfa (*Medicago sativa*). *Microbiology Resource Announcements* 8(38). <https://doi.org/10.1128/mra.00981-19>
 14. Jin, W, Zhang, Y, Su, X, Wang, R, Xie, Z, Wang, Y, Qiu, Y (2023) Development of Colloidal Gold Immunochromatography and Reverse-Transcription Loop-Mediated Isothermal Amplification Assays to Detect *Lychnis Mottle Virus*. *Plant disease* 107(5):1584-1592. <https://doi.org/10.1094/pdis-08-22-1970-re>
 15. Jin, W, Zhang, Y, Su, X, Xie, Z, Wang, R, Du, Z, Wang, Y, Qiu, Y (2023) Genetic diversity analysis of *lychnis mottle virus* and first identification of *Angelica sinensis* infection. *Heliyon* 9(6):e17006. <https://doi.org/https://doi.org/10.1016/j.heliyon.2023.e17006>
 16. Kusunoki, M, Hanada, K, Iwaki, M, Chang, MU, Dol, Y, Yora, K (1986) *Cycas necrotic stunt virus*, a new member of nepoviruses found in *Cycas revoluta* host range, purification, serology and some other properties. *Japanese Journal of Phytopathology* 52(2):302-311. <https://doi.org/10.3186/jjphytopath.52.302>
 17. Lim, M, Jana, S, Sivanesan, I, Park, H, Hwang, J, Park, Y, Jeong, BR (2013) Analysis of Genetic Variability Using RAPD Markers in *Paeonia* spp. Grown in Korea. *Korean Journal of Horticultural Science and Technology* 31. <https://doi.org/10.7235/hort.2013.12210>
 18. Lim, S, Kwon, S-Y, Lee, J-H, Cho, HS, Kim, H-S, Park, JM, Lee, S-H, Moon, JS (2019) Genomic detection and molecular characterization of two distinct isolates of *cycas necrotic stunt virus* from *Paeonia suffruticosa* and *Daphne odora*. *Virus Genes* 55(5):734-737. <https://doi.org/10.1007/s11262-019-01687-7>
 19. Lim, SH, Han, HS, Park, JH, Lee, J (2011) Methanol extract of peony root (*Peonia lactiflora*) and its ethyl acetate fraction attenuate heart and brain injury in a rat model of ischemia-reperfusion. *Journal of the Korean Society for Applied Biological Chemistry* 54(5):799-801. <https://doi.org/10.1007/BF03253163>
 20. Ochoa-Corona, FM (2003) Detection of *Cycas necrotic stunt virus* (CNSV) in post-entry quarantine stocks of ornamentals in New Zealand. *Phytopathology* 93:S67. <https://cir.nii.ac.jp/crid/1570854175402932096>
 21. Robertson, NL, Brown, KL, Winton, LM, Holloway, PS (2009) First Report of Tobacco rattle virus in Peony in Alaska. *Plant disease* 93(6):675-675. <https://doi.org/10.1094/pdis-93-6-0675b>
 22. Shaffer, CM, Gress, JC, Tzanetakis, IE (2019) First Report of *Cycas Necrotic Stunt Virus* and *Lychnis Mottle Virus* in Peony in the United States. *Plant disease* 103(5):1048-1048. <https://doi.org/10.1094/pdis-11-18-2089-pdn>
 23. Shaffer, CM, Michener, DC, Vlasava, NB, Chotkowski, H, Botermans, M, Starre, J, Tzanetakis, IE (2022) First Report of *Gentian Kobu-sho-Associated Virus* Infecting Peony in the United States and the Netherlands. *Plant disease* 106(4):1311. <https://doi.org/10.1094/pdis-06-21-1316-pdn>

24. Shaffer, CM, Michener, DC, Vlasava, NB, Chotkowski, H, Lamour, K, Stainton, D, Tzanetakis, IE (2021) The population structure of the secovirid lychnis mottle virus based on the RNA2 coding sequences. *Virus Research* 303. <https://doi.org/10.1016/j.virusres.2021.198468>
25. Shaffer, CM, Michener, DC, Vlasava, NB, Chotkowski, H, Tzanetakis, IE (2022) Population genetics of cycas necrotic stunt virus and the development of multiplex RT-PCR diagnostics. *Virus Research* 309:198655. <https://doi.org/10.1016/j.virusres.2021.198655>
26. Shaffer, CM, Vakić, M, Tzanetakis, IE (2021) First Report of Amazon Lily Mild Mottle Virus in Peony in the United States. *Plant disease* 105(1):236. <https://doi.org/10.1094/pdis-04-20-0915-pdn>
27. Shirima, RR, Maeda, DG, Kanju, E, Ceasar, G, Tibazarwa, FI, Legg, JP (2017) Absolute quantification of cassava brown streak virus mRNA by real-time qPCR. *Journal of Virological Methods* 245:5-13. <https://doi.org/10.1016/j.jviromet.2017.03.003>
28. Smith, CJ, Nedwell, DB, Dong, LF, Osborn, AM (2006) Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. *Environmental Microbiology* 8(5):804-815. <https://doi.org/10.1111/j.1462-2920.2005.00963.x>
29. Tang, J, Ward, L, Delmiglio, C (2020) Occurrence of Cycas Necrotic Stunt Virus in *Paeonia lactiflora* in New Zealand. *Plant disease* 104(5):1567-1567. <https://doi.org/10.1094/pdis-10-19-2277-pdn>
30. Uehara-Ichiki, T, Nakazono-Nagaoka, E, Ohashi, M, Sato, T, Hanada, K, Tatsuo, Y, Takao, Y, Murakami, Y, Kurosaki, F, Aoki, T, Fujikawa, T (2016) Detection of several plant viruses from medical herbs, *Rehmannia glutinosa* varieties, and their nucleotide sequences. *Japanese Journal of Phytopathology* 82(3):256. <https://doi.org/https://doi.org/10.3186/jjphytopath.82.192>
31. Uehara-Ichiki, T, Ohashi, M, Igarashi, M, Hanada, K, Hishida, A (2020) Two Plant Viruses Detected from Chinese Peony (*Paeonia lactiflora* Pall.) in Japan. *Annual Report of the Kanto-Tosan Plant Protection Society* 67(1):30-34. <https://doi.org/10.11337/ktpps.67.30>
32. Wylie, SJ, Luo, H, Li, H, Jones, MGK (2012) Multiple polyadenylated RNA viruses detected in pooled cultivated and wild plant samples. *Archives of Virology* 157(2):271-284. <https://doi.org/10.1007/s00705-011-1166-x>
33. Yoo, RH, Zhao, F, Lim, S, Igori, D, Lee, S-H, Moon, JS (2015) The complete nucleotide sequence and genome organization of lychnis mottle virus. *Archives of Virology* 160(11):2891-2894. <https://doi.org/10.1007/s00705-015-2501-4>
34. Yoon, J-Y, Soeng, C, Ju, H-J, Cho, I-S, Chung, BN (2024) First report of cycas necrotic stunt virus in a hybrid peony in South Korea. *Journal of Plant Pathology*. <https://doi.org/10.1007/s42161-023-01580-1>
35. Yu, S-J (1970) Anatomical Studies on the Classification of Cultivated Peony in Korea. *Korean Journal of Pharmacognosy* 1(3):81-92. <https://koreascience.kr/article/JAKO197003041163315.page>
36. Yu, X, Zhao, R, Cheng, F (2007) Seed germination of tree and herbaceous peonies: A mini-review. *Seed Sci. Biotechnol* 1:11-14.

Figures

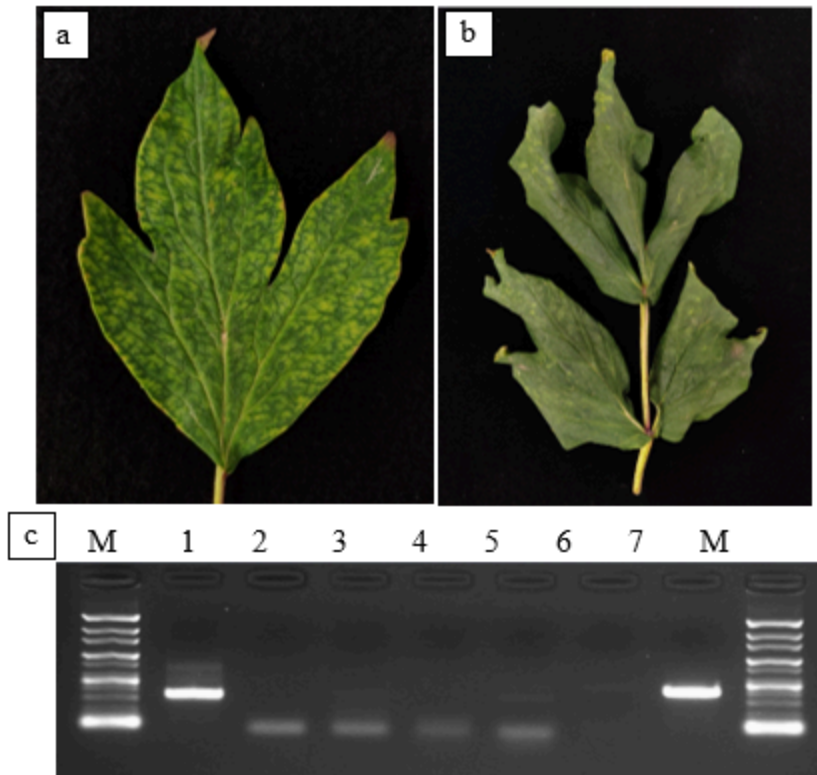


Figure 1

Collection of the symptomatic peony leaves and the detection of peony-infecting viruses by using RT-PCR. (a) the CNSV infected leaf. (b) the CNSV and LycMoV co-infected leaf. (c) the RT-PCR results from the co-infected leaf. 1 = cycas necrotic stunt virus (CNSV: 336 bp), 2 = tobacco rattle virus (TRV: 328 bp), 3 = amazon lily mild mottle virus (ALiMMV: 590 bp), 4 = citrus leaf blotch virus (CLBV: 281 bp), 5 = cucumber mosaic virus (CMV: 657 bp), 6 = gentian kobu-sho associated virus (GKaV: 574 bp), 7 = lychnis mottle virus (LycMoV: 412 bp), and M = 100 bp marker

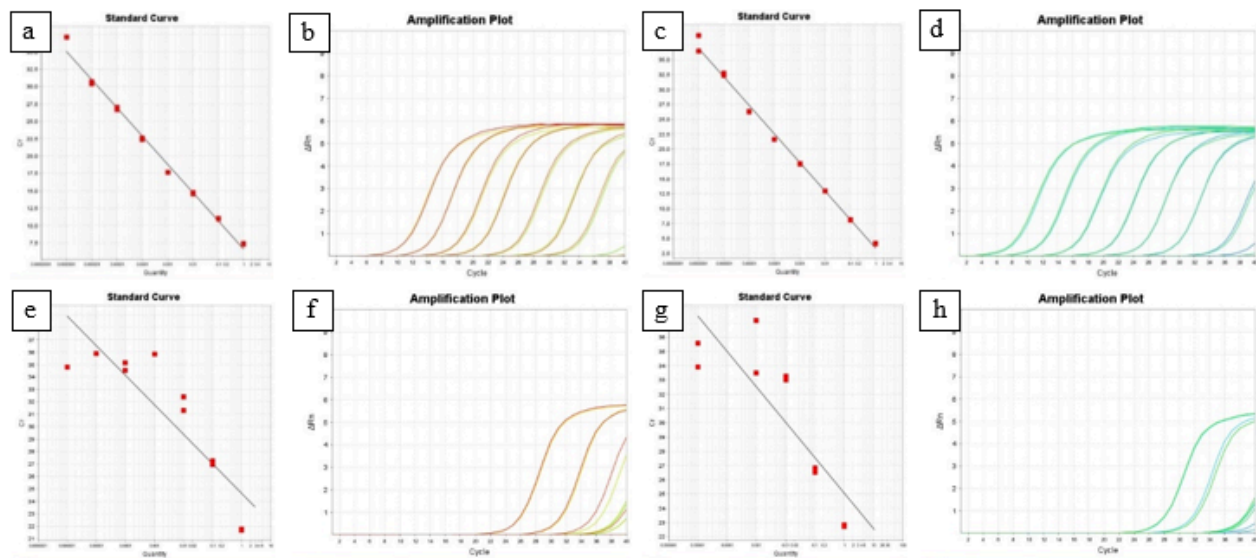


Figure 2

The standard curves and amplification plots (linear graph) of the different standard template types within the range of 1 ng/μl to 10⁻⁷ ng/μl. Standard curves of (a) the circular plasmid ($R^2 = 0.992$), (c) the linearized plasmid ($R^2 = 0.995$), (e) the total RNA ($R^2 = 0.781$), and (g) the transcript RNA ($R^2 = 0.722$). Amplification plots of (b) the circular plasmid, (d) the linearized plasmid, (f) the total RNA, and (h) the transcript RNA. The primer concentration was 10 pmol/μl and the annealing temperature was 55 °C. These experiments were conducted in the StepOne Real-Time PCR system (Applied Biosystems, United States)

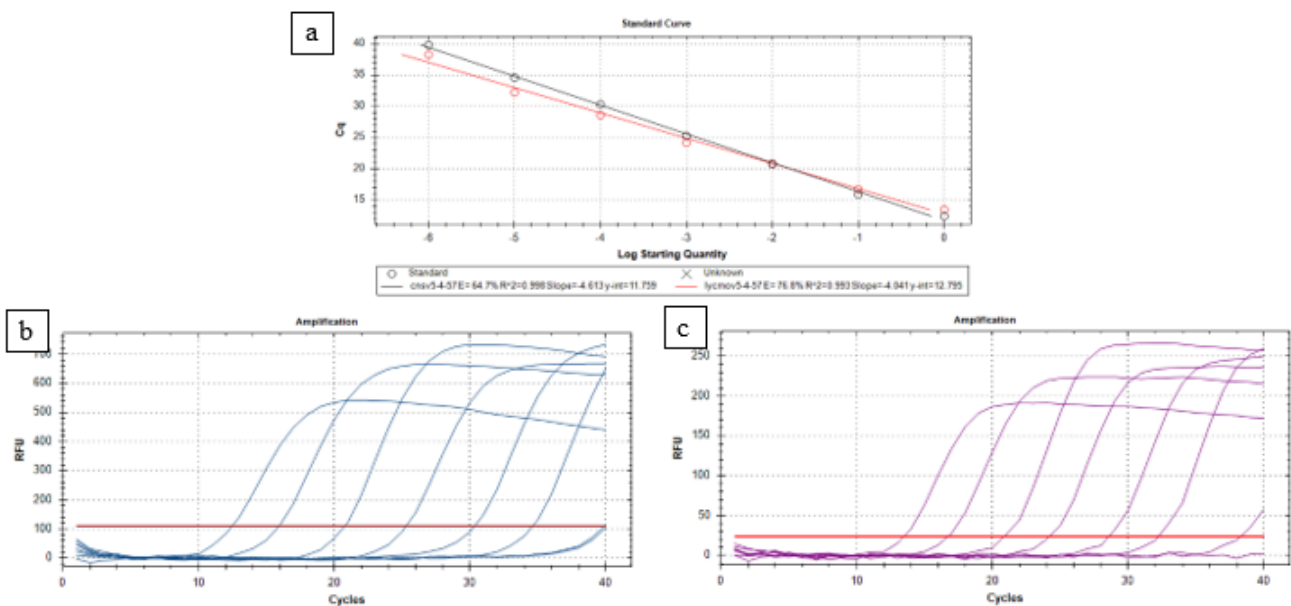


Figure 3

The standard curve, amplification plot, and detection limit of the optimized duplex probe-based qPCR assay conducted on cycas necrotic stunt virus (CNSV) and lychnis mottle virus (LycMoV). The primer concentration was 5 pmol/μl. The probe concentration was 4 pmol/μl. The annealing temperature was 57 °C. The standard range was from 1 ng/μl to 10⁻⁷ ng/μl. (a) The standard curve of CNSV (black) and LycMoV (red). (b) The amplification plot of CNSV and (c) LycMoV

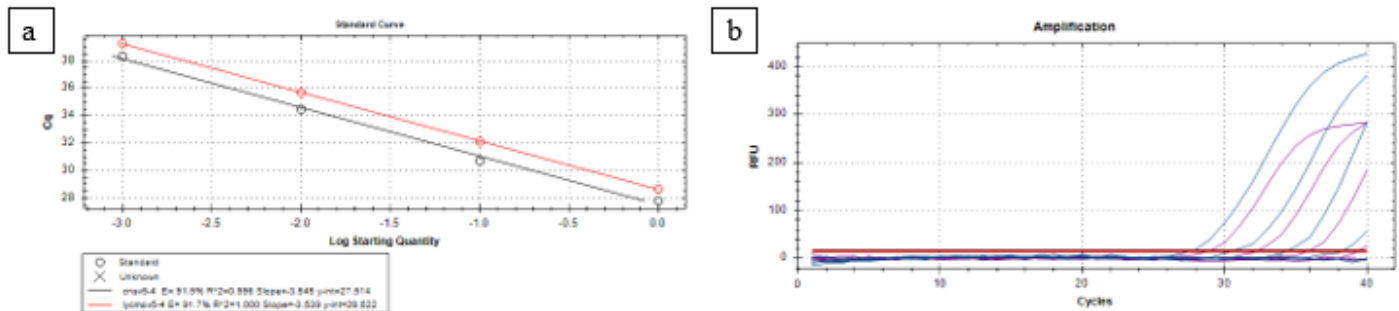


Figure 4

The standard curve, amplification plot, and detection limit of the duplex probe-based RT-qPCR experiment. (a) Amplification plot and (b) standard curve of simultaneous detection of cycas necrotic stunt virus (CNSV) and lychnis mottle virus (LycMoV). The primer concentration was 5 pmol/μl. The probe concentration was 4 pmol/μl. The annealing temperature was 57 °C. These were the same conditions derived from the optimized duplex probe-based qPCR experiment. The standard range was from 1 ng/μl to 10⁻⁴ ng/μl. The standard curve of CNSV target had an R² value of 0.996, while LycMoV target had an R² value of 1. The qPCR efficiencies were 91.5% for CNSV primer sets and 91.7% for LycMoV primer sets

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