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Application of Multiplex TaqMan Real-Time PCR Assay in Survey of Five Lily Viruses Infecting *Lilium* spp.

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Abstract: *Lily symptomless virus* (LSV), *Lily mottle virus* (LMOV), *Cucumber mosaic virus* (CMV), *Shallot yellow stripe virus* (SYSV), and *Plantago asiatica mosaic virus* (PIAMV) are five of the economically important viruses infecting lilies (*Lilium* spp.) worldwide. In order to prevent the occurrence and spread of these viruses, it is necessary to develop a rapid, effective, and sensitive detection method for the simultaneous detection and specific quantification of these viruses. In this study, specific primers and probes for multiplex TaqMan real-time PCR assays designed from conserved regions of the coat protein sequence of each virus were used for the simultaneous detection of these viruses in lilies (*Lilium* spp.). The optimal concentration of primers and probes and reaction annealing temperature were 20 μ M and 55.9 $^{\circ}$ C, respectively. The detection limits of the assay were 1.33×10^2 , 1.27×10^1 , 1.28×10^1 , 2.33×10^2 , and 2.01×10^2 copies $\cdot \mu$ L⁻¹ for LSV, LMOV, CMV, SYSV, and PIAMV, respectively. Specificity was determined using seven viral pathogens of lilies. Variability tests of intra- and inter-assays showed high reproducibility with coefficients of variation <2%. The multiplex TaqMan real-time PCR assay was used to detect these viruses from lily samples in China. In brief, our developed assay showed high specificity, sensitivity, and reproducibility for the simultaneous detection and differentiation of five lily-infecting viruses and can be used for certification and quarantine programs.

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Keywords: *Lilium* spp.; LSV; LMOV; CMV; SYSV; PIAMV; multiplex TaqMan real-time PCR; simultaneous detection

1. Introduction

Lilies (*Lilium* spp.) are among the most important ornamental plants worldwide and are also widely grown for their nutritional and medicinal value in Eastern Asia [1–3]. Lily cultivars are usually propagated vegetatively, resulting in virus transmission from generation to generation. Among viruses infecting lilies, *Lily symptomless virus* (LSV; genus *Carlavirus*, family *Flexiviridae*), *Cucumber mosaic virus* (CMV; genus *Cucumovirus*, family *Bromoviridae*), *Lily mottle virus* (LMOV; genus *Potyvirus*, family *Potyviridae*), *Shallot yellow stripe virus* (SYSV; genus *Potyvirus*, family *Potyviridae*), and *Plantago asiatica mosaic virus* (PIAMV; genus *Potexvirus*, family *Alphaflexiviridae*) are five economically important viruses [4–9]. LSV-infected plants show mild, pale vein clearing and mottle on leaves, which generally turn yellow fairly quickly [6,7]. LMOV can cause flower-color breaking, leaf mottle, leaf mosaic, chlorotic and yellow streaking, vein clearing, leaf curling, and narrowing [6,7]. CMV-infected lily leaves show chlorotic or yellow spotting, inter-veinal striping or vein-clearing and sometimes malformations [6,7]. Leaves infected with SYSV show yellow stripe symptoms [8]. When infected with PIAMV, leaves of infected plants are often brittle, flower production is declined, and the plants gradually die [9]. Mixed viral infections in lily plants can cause much more severe symptoms, including dwarfism [7]. In order to prevent the occurrence and spread of these viruses, it is essential to establish a rapid, effective, and sensitive detection method for the simultaneous detection and specific quantification of these viruses.

Many diagnostic approaches have been developed to detect viruses, such as serological methods relying on specific antibodies (enzyme-linked immunosorbent assay (ELISA), double-antibody sandwich-ELISA (DAS-ELISA), immunochromatographic strip test, etc.) and molecular biology methods relying on specific primers/probes (reverse transcription-polymerase chain reaction (RT-PCR), real-time PCR, loop-mediated isothermal amplification (LAMP), etc.) [10–17]. ELISA, uniplex RT-PCR, and LAMP have been used to detect lily viruses [18–22]; however, these techniques can only be used to detect one virus per reaction and are time-consuming, laborious, and expensive when several viruses have to be detected in numerous samples [23]. These limitations can be overcome by using multiplex TaqMan real-time PCR assay, which enables the simultaneous detection and identification of several viruses in a single reaction [14]. Multiplex TaqMan real-time PCR has been applied widely for pathogen detection in humans [14,24–27] and animals [10,28–35]. Recently, this method has also been used to detect viruses in sweet potatoes, pome fruit trees, stone fruit trees, and fig trees [36–40]. However, this method has not been used for the simultaneous detection of LSV, LMoV, CMV, SYSV, and PIAMV in lily plants.

In this study, a set of specific primers and probes were designed for the detection of five economically important viruses (LSV, LMoV, CMV, SYSV, and PIAMV) infecting lily plants. Then, an efficient multiplex TaqMan real-time PCR was developed for the simultaneous detection and differentiation of these lily viruses.

2. Materials and Methods

2.1. Materials

Fresh leaves from lily samples grown in greenhouses at the Chinese Academy of Agricultural Sciences (Beijing, China), which were confirmed to be infected with LSV, LMoV, CMV, SYSV, and PIAMV by RT-PCR and sequencing, were used to establish and optimize the multiplex TaqMan real-time PCR assay.

2.2. RNA Extraction and Reverse Transcription

Total RNA was extracted from lily leaves using an RNAPrep pure Plant Kit (Tiangen, Beijing, China), according to the manufacturer's protocol. First-strand cDNA was synthesized using a SuperScript III reverse transcription kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Briefly, 5 µg total RNA was combined with 1 µL oligo (dT)₂₀ (50 µM) and 1 µL dNTP mix (10 mM), and nuclease-free water was added to a total volume of 13 µL. The mixture was heated at 65 °C for 5 min and incubated on ice for 1 min. The volume was increased to 20 µL by the addition of 4 µL 5 × first-strand buffer, 1 µL RNaseOUT (40 U), and 1 µL Superscript III reverse transcriptase (200 U). After incubation at 50 °C for 60 min, 1 µL RNase H (2 U) was added, and incubation was continued at 37 °C for 20 min.

2.3. Design of Primers and TaqMan Probes

Sequences of the complete genomes of LSV (accession numbers: HM222522, AJ516059, AM263208, AM422452, and GU440579), LMoV (accession numbers: AB570195, GU440578, AM048875, GU440579, NC_005288, MT795719, and HM222521), CMV (accession numbers: AB506797, AB506800, AJ495841, and AB049568), SYSV (accession numbers: MK127536 and AJ865076), and PIAMV (accession numbers: AB360790, AB360791, AB360792, LC592411, KU159093, LN794199, KT717325, KU159089, and KX245539) were retrieved from the GenBank database, and conserved regions of each virus were identified using ClustalW (Available online: <http://clustalw.ddbj.nig.ac.jp/> (accessed on 16 January 2021)). Virus-specific primers and probes were designed according to the identified conserved regions using Beacon Designer 8.0 (Premier Biosoft, Palo Alto, CA, USA) (Table 1).

Table 1. Primers and probes used in this study.

Virus	Primer/Probe	Target Gene	Sequence (5′–3′)	Product Length (bp)
LSV	LSV-F	CP	GCGTCGTATCTAACAACA	184
	LSV-R		GCTCCATTCTCAAAGTCA	
	LSV-Probe		CY5-CAAGGAACGCCGAAGTCTC-BHQ3	
LMOV	LMOV-F	CP	CAGTGAAAGACGAGTATG	88
	LMOV-R		GAGGTGCCATTCTCTATG	
	LMOV-Probe		FAM-CAGACCATTTCATTGCGAGAGCC-BHQ1	
CMV	CMV-F	CP	GACAGTCCGTAAAGTTCC	105
	CMV-R		GATGCAGCGTACTGATAA	
	CMV-Probe		Texas Red-TATCCGTTGCCGCCATCTCT-BHQ2	
SYSV	SYSV-F	CP	GCTTGGATGGTAACATAAG	80
	SYSV-R		CGTGTGATGATCCTTATTC	
	SYSV-Probe		CY5.5-AGAACGACATACAGCAGCCGABHQ2	
PIAMV	PIAMV-F	CP	CCAACATCAAGTTCGAAC	153
	PIAMV-R		CGAAGAGGTTTAGGGATC	
	PIAMV-Probe		HEX-CGTCTCATTGGCAGTTACTTCGTC-BHQ1	

2.4. Optimization of the Multiplex TaqMan Real-Time PCR Assay

The primers and probes used in these assays were listed in Table 1. All reactions were performed on a Bio-Rad CFX96™ Real-time System (Bio-Rad, Hercules, CA, USA). For uniplex real-time PCR amplifying LSV, LMOV, CMV, SYSV, and PIAMV, the reaction mixture contained 0.4 µL forward primer (20 µM), 0.4 µL reverse primer (20 µM), 0.4 µL TaqMan probe (20 µM), 10 µL 2 × PerfectStart® II Probe qPCR SuperMix (TransGene, Beijing, China), 2 µL template, and nuclease-free water for a total volume of 20.0 µL. The thermal cycling procedure was initiated at 94 °C for 30 s, followed by 40 cycles at 94 °C for 5 s and 56 °C for 30 s.

Based on the established uniplex real-time PCR, the multiplex real-time PCR was optimized by varying a single parameter while other parameters were maintained. The multiplex real-time PCR was optimized by varying the annealing temperature and the concentration of primers and probes. Different annealing temperatures were set from 52.0 to 62.0 °C (52.0 °C, 52.7 °C, 54.0 °C, 55.9 °C, 58.4 °C, 60.3 °C, 61.4 °C, and 62.0 °C), and the concentrations of primers and probes ranged from 10 to 30 µM (10 µM, 15 µM, 20 µM, 25 µM, and 30 µM). The optimal PCR conditions were selected based on amplification efficiency determined by the C_q (Cycle of quantification) value and the fluorescence intensity. All reactions were conducted in triplicates.

2.5. Construction of Standard Plasmids and Standard Curves

Conventional RT-PCR assays were used to produce the standard fragments of the target viruses using the primers listed in Table 1 (LSV-F/LSV-R for LSV, LMOV-F/LMOV-R for LMOV, CMV-F/CMV-R for CMV, SYSV-F/SYSV-R for SYSV, and PIAMV-F/PIAMV-R for PIAMV). PCR amplification reactions were performed in a final volume of 50 µL containing 1.5 µL each primer (10 µM), 2 µL cDNA, 25 µL 2 × KAPA HiFi HotStart Ready Mix (KAPA Biosystems, Wilmington, WA, USA), and 20 µL double-distilled water. The thermal cycling conditions were as follows: 95 °C for 3 min, followed by 35 cycles of 98 °C for 20 s, 56 °C for 15 s, 72 °C for 30 s, and a final extension at 72 °C for 2 min. The PCR products were electrophoresed in a 2% agarose gel in 0.5 × TBE buffer. PCR products were cloned into the pTOPO vector (Transgen, Beijing, China) to construct recombinant

plasmids and sequenced with vector primers at Sangon Biotech (Shanghai, China). The concentrations of the recombinant plasmids were quantified using a Nano Drop (Thermo Scientific, Waltham, MA, USA), and the copy number was calculated as follows: number of copies = [amount (ng) $\times$ 6.022 $\times$ 10²³]/[length (plasmid + insert) $\times$ 1 $\times$ 10⁹ $\times$ 650] [41]. Standard curves were obtained using serial ten-fold dilutions of the recombinant plasmids of corresponding viruses.

2.6. Specificity of the Multiplex Real-Time PCR

In order to evaluate the specificity of this multiplex real-time PCR assay developed in this study, cDNAs of two other lily viruses, including *Strawberry latent ring spot virus* (SLRSV; genus *Stralarivirus*, family *Secoviridae*) and *Arabidopsis mosaic virus* (ArMV; genus *Nepovirus*, family *Secoviridae*), were used as templates for amplification with this multiplex system. The cDNAs of LSV, LMoV, CMV, SYSV, and PIAMV served as positive controls, and the nuclease-free water was used as a negative template control.

2.7. Analytical Sensitivity

To evaluate the detection limits of the multiplex real-time PCR compared with the uniplex real-time PCRs, 10-fold serial dilutions of the mixture of five recombinant plasmids, ranging from 1.33 $\times$ 10⁹ to 1.33 $\times$ 10⁰ copies $\cdot$ μ L⁻¹, 1.27 $\times$ 10⁹ to 1.27 $\times$ 10⁰ copies $\cdot$ μ L⁻¹, 1.28 $\times$ 10⁹ to 1.28 $\times$ 10⁰ copies $\cdot$ μ L⁻¹, 2.33 $\times$ 10⁹ to 2.33 $\times$ 10⁰ copies $\cdot$ μ L⁻¹, and 2.01 $\times$ 10⁹ to 2.01 $\times$ 10⁰ copies $\cdot$ μ L⁻¹ for LSV, LMoV, CMV, SYSV, and PIAMV, respectively, were used as templates. Samples with a Cq value greater than 35 were considered negatives.

2.8. Reproducibility

To evaluate the reproducibility of the multiplex real-time PCR, three different concentrations of 10⁷, 10⁵, and 10³ copies $\cdot$ μ L⁻¹ of standard plasmids were chosen to perform in triplicate on different days. The final coefficient of variation (CV) was calculated to assess intra- and inter-assay variation.

2.9. Survey of Lily Viruses by the Multiplex Real-Time PCR Assay

To further evaluate the efficiency of the multiplex real-time PCR assay, 92 lily samples that showed symptoms of leaf mottle, leaf mosaic, leaf curling, chlorotic and yellow streaking, or flower color breaking were collected from lily production fields in China for the detection of LSV, LMoV, CMV, SYSV, and PIAMV using the developed multiplex TaqMan real-time PCR assay. RNA extraction and reverse transcription were performed using the method described in Section 2.2. Multiplex real-time PCR amplification reactions were performed in a final volume of 20 μ L containing 0.4 μ L of each of 20 μ M LSV, LMoV, CMV, SYSV, and PIAMV primers and probes, 10 μ L 2 $\times$ PerfectStart[®] II Probe qPCR SuperMix (TransGene, Beijing, China), 2 μ L template, and nuclease-free water was added to a total volume of 20.0 μ L. The PCR conditions were determined as follows: 94 $^{\circ}$ C for 30 s, followed by 40 cycles at 94 $^{\circ}$ C for 5 s, 55.9 $^{\circ}$ C for 30 s. At the same time, the uniplex real-time PCR was performed using the same samples, and the results of the two methods were compared.

2.10. Statistical Analysis

Data analysis and graphing were performed using GraphPad Prism (version 8 for Windows, GraphPad Software, San Diego, CA, USA) and using Jvenn (Available online: <http://jvenn.toulouse.inra.fr/app/example.html> (accessed on 27 September 2021)) [42].

3. Results

3.1. Optimization of the Multiplex TaqMan Real-Time PCR Assay

Figure 1 shows the optimum concentrations of primers and probes for the multiplex TaqMan real-time PCR assay, which are 20, 30, 20, 20, and 20 μ M for LSV, LMoV, CMV, SYSV, and PIAMV, respectively. The results showed the optimum annealing temperatures

for LSV, LMoV, CMV, SYSV, and PIAMV amplification were 58.4, 55.9, 58.4, 55.9, and 55.9 °C, respectively (Figure 2). Based on the comparison of reaction performance, 20 µM and 55.9 °C were selected to be the optimal primers and probes concentration and reaction annealing temperature.

Figure 1. Optimization of the concentration of primers and probes for multiplex TaqMan real-time PCR assay to detect LSV (A), LMoV (B), CMV (C), SYSV (D), and PIAMV (E).

Figure 2. Optimization of the annealing temperature for multiplex TaqMan real-time PCR assay to detect LSV (A), LMoV (B), CMV (C), SYSV (D), and PIAMV (E).

3.2. Standard Curves of the Multiplex TaqMan Real-Time PCR Assay

Fragments of the expected size for LSV, LMoV, CMV, SYSV, and PIAMV were amplified specifically using virus-specific primer pairs (Figure 3) and were cloned into pTOPO vector (Transgen, Beijing, China) to obtain recombinant plasmids pTOPO-LSV, pTOPO-LMoV, pTOPO-CMV, pTOPO-SYSV, and pTOPO-PIAMV. The stock of these plasmids had concentrations of 1.33×10^{10} (pTOPO-LSV), 1.27×10^{10} (pTOPO-LMoV), 1.28×10^{10} (pTOPO-CMV), 2.33×10^{10} (pTOPO-SYSV), and 2.01×10^{10} copies· μL^{-1} (pTOPO-PIAMV). Standard curves were constructed using 10-fold serial dilutions of the recombinant plasmids. The results showed that the correlation coefficients (R^2) of the five standard curves were above 0.98 (Figure 4), indicating that there was a strong linear relationship between the $\log_{10}$ of the input number of copies and the Cq value of each standard.

Figure 3. Determination of specificity of five primer pairs by uniplex RT-PCR to detect LSV (A), LMoV (B), CMV (C), SYSV (D), and PIAMV (E). Lane M, 100 bp plus DNA ladder.

Figure 4. Standard curves obtained by multiplex TaqMan real-time PCR assay for LSV (A), LMoV (B), CMV (C), SYSV (D), and PIAMV (E).

3.3. Specificity of the Multiplex TaqMan Real-Time PCR Assay

The results showed that only the positive samples of LSV, LMoV, CMV, SYSV, and PIAMV showed the amplification curve (Figure 5A–E,H), while neither nuclease-free water nor the two non-targeted viruses showed the amplification curve (Figure 5F,G,I).

Figure 5. Specificity of multiplex TaqMan real-time PCR. The specific fluorescent signals were detected in the positive samples of LSV (A), LMoV (B), CMV (C), SYSV (D), and PIAMV (E) and mixed samples (H). No cross-reactions were detected from ArMV (F) and SLRSV (G) and ddH₂O (I).

3.4. Sensitivity of the Multiplex TaqMan Real-Time PCR Assay

Sensitivity testing showed that detection limits were 1.33×10^2 for LSV, 1.27×10^1 for LMoV, 1.28×10^1 for CMV, 2.33×10^2 for SYSV, and 2.01×10^2 for PIAMV in both multiplex TaqMan real-time PCR and uniplex TaqMan real-time PCR (Figures 6 and 7). These results indicated that the sensitivity of the multiplex TaqMan real-time PCR was the

same as that of each uniplex TaqMan real-time PCR assay and that the multiplex TaqMan real-time PCR was suitable for the simultaneous detection of the five viruses.

Figure 6. Sensitivity of the multiplex TaqMan real-time PCR assay for LSV (A), LMoV (B), CMV (C), SYSV (D), and PIAMV (E). 1–10, ten-fold serial dilutions of plasmid standard, with the concentrations from 1.33×10^9 to 1.33×10^0 copies·μL⁻¹, 1.27×10^9 to 1.27×10^0 copies·μL⁻¹, 1.28×10^9 to 1.28×10^0 copies·μL⁻¹, 2.33×10^9 to 2.33×10^0 copies·μL⁻¹, and 2.01×10^9 to 2.01×10^0 copies·μL⁻¹ for LSV, LMoV, CMV, SYSV, and PIAMV, respectively.

Figure 7. Sensitivity of the uniplex TaqMan real-time PCR assay for LSV (A), LMoV (B), CMV (C), SYSV (D), and PIAMV (E). 1–10, ten-fold serial dilutions of plasmid standard, with the concentrations from 1.33×10^9 to 1.33×10^0 copies·μL⁻¹, 1.27×10^9 to 1.27×10^0 copies·μL⁻¹, 1.28×10^9 to 1.28×10^0 copies·μL⁻¹, 2.33×10^9 to 2.33×10^0 copies·μL⁻¹, and 2.01×10^9 to 2.01×10^0 copies·μL⁻¹ for LSV, LMoV, CMV, SYSV, and PIAMV, respectively.

3.5. Repeatability of the Multiplex TaqMan Real-Time PCR Assay

Different standard plasmids concentrations (10^7 , 10^5 , and 10^3 copies· μL^{-1}) were chosen to evaluate the intra- and inter-assay repeatability. The CV values were almost less than 1%, with a few values ranging from 1% to 2% (Table 2) in the intra- and inter-assays, indicating that the assay has high repeatability and reliability.

Table 2. Intra- and inter-assay reproducibility of multiplex TaqMan real-time PCR.

Plasmid	Concentration (Copies·μL ⁻¹)	Intra-Assay Cq Value			Inter-Assay Cq Value			
		Mean	SD	CV%	Mean	SD	CV%	
LSV	1.33×	10 ⁷	17.05	0.29	1.68	17.68	0.02	0.11
		10 ⁵	25.80	0.30	1.15	26.74	0.07	0.27
		10 ³	31.17	0.07	0.23	31.53	0.18	0.58
LMoV	1.27×	10 ⁷	17.41	0.02	0.12	16.39	0.21	1.30
		10 ⁵	23.84	0.12	0.49	23.97	0.22	0.93
		10 ³	30.65	0.16	0.52	31.15	0.13	0.42
CMV	1.28×	10 ⁷	18.40	0.21	1.14	18.56	0.08	0.41
		10 ⁵	26.20	0.20	0.76	26.23	0.20	0.76
		10 ³	30.89	0.14	0.44	30.64	0.19	0.61
SYSV	2.33×	10 ⁷	16.02	0.15	0.94	16.08	0.09	0.54
		10 ⁵	24.30	0.05	0.19	24.48	0.07	0.27
		10 ³	30.59	0.04	0.14	31.21	0.08	0.27
PIAMV	2.01×	10 ⁷	15.80	0.28	1.77	15.51	0.12	0.77
		10 ⁵	24.29	0.46	1.89	23.61	0.24	1.03
		10 ³	31.65	0.55	1.74	32.09	0.08	0.25

3.6. Application of Multiplex TaqMan Real-Time PCR Assay in Survey of Lily Viruses

The results of the multiplex TaqMan real-time PCR assay showed that all samples were infected with at least three viruses (Table S1). LSV, LMoV, CMV, SYSV, and PIAMV were detected in 92 (100.00%), 61 (66.30%), 92 (100.00%), 76 (82.61%), and 72 (78.26%) of the 92 samples, respectively (Figure 8A). There were 8 samples co-infected with three viruses, 51 samples co-infected with four viruses, and 33 samples co-infected with five viruses (Figure 8B). All collected samples were further tested using the uniplex TaqMan real-time PCR, and the results were consistent with those of the multiplex TaqMan real-time PCR assay (data not shown). Thus, the multiplex TaqMan real-time PCR assay developed in this study was confirmed to be a rapid and accurate detection method.

Figure 8. Detection of five viruses in lily plants in China using multiplex TaqMan real-time PCR assay. (A) Venn diagram illustrating the number of samples infected by multiplex TaqMan real-time PCR assay. (B) Number of samples infected with different viruses.

4. Discussion

Primer design is the most important step in establishing an efficient multiplex TaqMan real-time PCR assay. Ideally, primers should be specific, have low or no interference in the amplification reaction [38]. In this study, considering the wide genetic diversity of LSV, LMoV, CMV, SYSV, and PIAMV, virus-specific primers were designed according to conserved regions in each viral genome. Using the final primers, five expected amplicons (184 bp for LSV, 88 bp for LMoV, 105 bp for CMV, 80 bp for SYSV, and 153 bp for PIAMV) were produced (Figure 3). And, using this multiplex TaqMan real-time PCR, only the positive samples of LSV, LMoV, CMV, SYSV, and PIAMV showed the amplification curve (Figure 5), revealing that this newly developed assay was highly specific.

The optimization of PCR conditions is essential for efficient PCR amplification. In this study, serial PCRs were conducted to determine the optimal concentration of primers and probes and annealing temperature. The results showed the C_q value and the fluorescence intensity were highest when the concentration of primers and probes and the reaction annealing temperature were 20 µM and 55.9 °C, respectively (Figures 1 and 2). Generally, the presence of more than one pair of primers in the same reaction mix may limit the sensitivity or cause preferential amplification of specific targets [43], and the sensitivity of the multiplex PCR is usually about 10-fold lower than that of a uniplex PCR [36]. Fortunately, the sensitivity of the optimized multiplex TaqMan real-time PCR assay was the same as that of each uniplex assay in our previous study, indicating that a desired primer design and the proper optimization of the multiplex TaqMan real-time PCR assay were developed.

Viral diseases frequently occur in lily plants, and they affect bulb and cut-flower production [4–9]. In this study, 92 lily samples in China were tested using the developed multiplex TaqMan real-time PCR assay. The results showed all samples were infected with at least three viruses (Table S1, Figure 8). The high occurrence of viruses infecting lilies in China could be due to the use of virus-infected bulbs for propagation and the lack of preventive virus vector control measures. Hence, it is urgent to use virus-free propagation materials and to take extreme precautions to produce virus-free lily bulbs.

In conclusion, an effective multiplex TaqMan real-time PCR assay was established for the simultaneous detection and differentiation of LSV, LMoV, CMV, SYSV, and PIAMV in lilies. This assay will be useful for routine molecular diagnosis and epidemiological studies of these viruses.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy12010047/s1>, Table S1: Survey of viral infection in lily plants in China using the multiplex TaqMan real-time PCR assay.

Author Contributions: Validation, L.X. and M.S.; formal analysis, M.S.; investigation, L.X. and M.S.; resources, L.X.; data curation, L.X. and J.M.; writing—original draft preparation, L.X.; writing—review and editing, J.M.; project administration, J.M.; funding acquisition, L.X. and J.M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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