Reverse Transcription Polymerase Chain Reaction-based System for Simultaneous Detection of Multiple Lily-infecting Viruses

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A detection system based on a multiplex reverse transcription (RT) polymerase chain reaction (PCR) was developed to simultaneously identify multiple viruses in the lily plant. The most common viruses infecting lily plants are the cucumber mosaic virus (CMV), lily mottle virus (LMoV), lily symptomless virus (LSV). Leaf samples were collected at lily-cultivation facilities located in the Kangwon province of Korea and used to evaluate the detection system. Simplex and multiplex RT-PCR were performed using virus-specific primers to detect single- or mixed viral infections in lily plants. Our results demonstrate the selective detection of 3 different viruses (CMV, LMoV and LSV) by using specific primers as well as the potential of simultaneously detecting 2 or 3 different viruses in lily plants with mixed infections. Three sets of primers for each target virus, and one set of internal control primers were used to evaluate the detection system for efficiency, reliability, and reproducibility.

Keywords : bulb plant, *Lilium*, siberia, sorbonne

The lily is one of the most popular flower crops cultivated throughout the world, including Korea. The genus *Lilium* contains about 4,500 species, including the commercially important Easter lily (*Lilium logiflorum*) as well as the Asiatic and Oriental hybrid lily plants (Sharma et al., 2005). Among the lily-infecting viruses, cucumber mosaic virus (CMV), lily mottle virus (LMoV), and lily symptomless virus (LSV) are considered to be challenging viral pathogens for different varieties of lily plants. Specifically, viral infections can be spread by aphids and are known to occur in many cultivation regions of the world (Asjes, 2000; Choi and Ryu, 2003). Notably, LSV, a species of the genus *Carlavirus*, is currently one of the most prevalent viruses infecting lily plants throughout the world. CMV and LMoV are prevalent in Asiatic and Oriental lily cultivars in India (Sharma et al., 2005) and can damage the growth of lily

plants in combination with other viruses. Other viruses such as the arabis mosaic virus (ArMV), lily virus X (LVX), plantago asiatica mosaic virus (PIAMV), strawberry latent ringspot virus (SLRV), tobacco rattle virus (TRV), and tomato ringspot virus (TRSV) have also been reported to infect lily plants (Asjes, 2000; Komatsu et al., 2008; Sharma et al., 2005). When lily plants become infected with more than one virus, the symptoms expressed tend to be more severe, such as vein clearing, leaf rolling, spotting, mosaic patterns, and yellow streaking. Symptom variation due to coinfection with other viruses and seasonal changes also causes difficulties in the visual evaluation of diseased plants (Kong et al., 2009), and viruses can easily be maintained in bulb stocks, depending on how vegetative propagation is practiced (e.g., tissue cultures and bulb scaling). A number of detection techniques based on biological, cytological, serological, and structural properties investigation have been developed for detecting viruses. Hsu et al. (1995) performed dot-blot immunoassay (DBIA), a tissue-blot immunoassay (TBIA), and an enzyme-linked immunosorbent assay (ELISA) for the detection of LSV and compared the results. LMoV has been reliably detected in the bulb of Asiatic hybrids by using a double-antibody-sandwich (DAS) ELISA (Derks et al., 1997), but the method failed in *L. longiflorum* and Oriental hybrids. There is an apparent difference between the detection efficiencies of each method. Although DAS-ELISA has been used for virus detection because it can test large amount of bulbs per season, other precise and reliable methods have also been evaluated. The PCR amplification of gene segments based on viral genome sequences is a very sensitive technique for the screening and identification of viruses, which are not easy to diagnose by other known methods, especially for bulb plants. Niimi et al. (2003) reported that the detection of lily viruses was more efficient by RT-PCR than ELISA. Sharma et al. (2005) reported that RT-PCR was reliable for detecting viral infections regardless of the growth stage and whether leaves were sampled at early or flowering stages. Importantly, serological methods can sometimes produce non-specific reactions and their low sensitivity can preclude the identification of a virus when a low titer of antiserum is used

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(Sato et al., 2002). Currently, mixed viral infections in Cucurbitaceae crops are detected using multiplex RT-PCR (Shimomoto and Takeuchi, 2006), which is a highly sensitive method for detecting viruses (in picogram amounts) and identifying virus species in one reaction (Kuroda et al., 2002). A modified PCR method has been reported that incorporates a dual priming oligonucleotide (DPO) system that differs structurally and functionally from the conventional primer system by including a poly(I) linker between 2 segments of primer sequences (Chun et al., 2007). We developed a highly sensitive molecular detection system for lily-infecting viruses by using DPO primers in multiplex RT-PCR. To facilitate the study of viral infection in lily plants and develop a prevention system, we developed a multiplex RT-PCR system that amplifies 3 viral genes, CMV, LMoV, and LSV, and the 18S ribosomal RNA (rRNA) internal control in one reaction.

Field samples were collected at lily-cultivation facilities located in the Kangwon province of Korea. Leaves showing symptoms were collected and prepared for total RNA extraction (Fig. 1). The following samples were collected: *Lilium* Oriental hybrid cultivars such as ‘Siberia’, ‘Sorbonne’, ‘Donato’, and ‘Valparaiso’. Total RNA extracts were prepared using the Plant RNeasy mini kit (QIAGEN, Germany), and total RNA was quantified by a UV spectrophotometer (Thermo Scientific, USA). Dried leaf samples individually infected with CMV, LMoV, and LSV were distributed from the Plant Virus Bank (Seoul Women’s University, Korea), and used as a positive control for each virus in the evaluation of the multiplex RT-PCR system. Primers (Bionics, Korea) for simplex RT-PCR of virus-infected lily plants were designed and synthesized before multiplexing reactions were performed (Table 1). Specific primer pairs were synthesized according to the target sequences of CMV, LMoV, and LSV in order to anneal to specific sequences in each virus. As a positive internal con-

Fig. 1. Symptoms caused by lily-infecting viruses. Each leaf was shown to be virus infected with (A) CMV, (B) LMoV, (C) LSV, (D) LMoV and LSV, (E) CMV, LMoV and LSV.

trol during PCR, conserved sequences of 18S rRNA were targeted as regions for primer annealing. These primers were designed to have similar annealing melting temperatures to prevent the formation of primer dimers or secondary structure. The target regions for detection via simplex RT-PCR are shown in Table 1. Reverse transcription (RT) was performed in a 20 µl reaction at 42 °C for 1 h in 20 mM Tris-Cl buffer (pH 8.0) containing sample RNA (approximately 1 µg), 5 mM MgCl₂, 50 mM KCl, 1.0 mM of each of the 4 dNTPs, 50 pmol reverse primer for simplex PCR or 100 pmol random hexamers (Roche, Swiss) for multiplex PCR, 1 unit of RNase inhibitor (Takara, Japan), and 2.5 units MuLV reverse transcriptase (Qbiogene, USA). Reverse transcriptase was inactivated at 92 °C for 10 minutes after RT. Simplex RT-PCR was performed using each primer pair to evaluate their specificity and optimize the PCR conditions. Two microliters of complementary DNA (cDNA) synthesized from of RT reaction was mixed with 2×master-

Table 1. Primers used in simplex and multiplex RT-PCR for lily-infecting viruses

Primer	Sequence 5'-3'	Expected amplicon size (bp)	Target	T _m (°C)	Accession No.
LMoV-F	GACCCAACCGAATATTCATTGCCC[IIIIITGTCGACCCAC]	350	Viral protein genome-linked	55	NC005288.1
LMoV-R	TCCTTAGGCAACTCTGCAGC[IIIIICCATGACTG]				
CMV-F	ACGTCTCTRTTCAACATCAATGAA[IIIIAGCCTCCCAC]	285	RNA1a	55	AF127976.1
CMV-R	GTCTAGACAATCGAGAGTTTCACA[IIIIIGGAGGGCACC]				
LSV-F	CAACCACTGAACAAATGGCCA[IIIIIGCGTCAGAC]	205	Coat protein	55	AM263208.1
LSV-R	GCGTGCTTCTTCATGATCG[IIIIATGGAGTCGAC]				
18S-F	GACGGAGAATTAGGGTTCGATT[IIIIIGAGGGAGCC]	813	18S rRNA	55	AF206894.1, AF206952
18S-R	CTCCACTCCTGGTGGTGCC[IIIIIGTCAATTCC]				

*Sequences in brackets indicate primers designated for multiplex RT-PCR

**I represents inosine

mix (Seegene Co, Korea) containing 10 pmol of forward and reverse primers designed for simplex RT-PCR. To adjust the compatibility of primer pairs, different combinations of primer pairs were tested using virus-infected samples before performing multiplex RT-PCR. The primers for multiplex RT-PCR were designed according to the DPO technique. With the exception of using 50 pmol LSV-specific primers, multiplex RT-PCR was performed using the same conditions used for simplex RT-PCR. Both simplex and multiplex RT-PCR conditions were adjusted for a volume of 20 μ l. PCR was performed in a thermal cycler (BIO-RAD, USA) using the following conditions: predenaturation at 94°C for 15 minutes before starting the PCR cycle; 35 PCR cycles consisting of 30 seconds at 94°C, 60 seconds at 55°C, 60 seconds at 72°C; and a final extension at 72°C for 10 minutes. The amplification (5 μ l) products were then examined by electrophoresis on 2.0% agarose gels in half-strength TAE buffer at 50V for 60 minutes.

Lily leaves from ‘Siberia’ showing typical symptoms of virus infection were collected from cultivation fields and tested using simplex RT-PCR with the corresponding virus-specific primers listed in Table 1 (Fig. 1). Conventional primers that were designed for single target virus detection were used in simplex RT-PCR. The resulting DNA amplicons with the expected size of each virus were observed in lily samples using virus-specific primer pairs, whereas no specific bands were detected in the negative control samples containing DNA from healthy lily plants. Compared to the positive controls, the RT-PCR amplicons generated from RNA genome sequences of LMoV, CMV, and LSV showed specific bands corresponding to 350, 285, and 205 bp, respectively (Fig. 2). Amplified fragments were confirmed by sequencing, and determined nucleotide sequences were compared to reported sequences in database (National Center for Biotechnology Information). Based on the gel electrophoresis of simplex RT-PCR products, samples were infected with either each of the 3 viruses or a mixture, indicating that specific primer pairs can distinctively amplify

Fig. 2. Simplex RT-PCR detection of infection in lily samples by using corresponding virus-specific primers. The lane numbers indicate the same lily sample. The positive control (+) was derived from virus-infected dried leaves. (–) represents a healthy sample used as the negative control.

the corresponding cDNA of each virus (Fig. 2). Fig. 2A shows that the samples in lanes 3, 5, 6, 7, and 8 were infected with LMoV as evidence by the size of the 350-bp RT-PCR amplicons. Samples in lanes 2, 4, and 8 were determined to be CMV-specific RT-PCR bands of 285 bp (Fig. 2B). Samples in lanes 1, 4, 5, 6, 7, and 8 were infected with LSV based on the 205-bp RT-PCR amplicons (Fig. 2C). Notably, samples in several lanes were infected with more than one virus (lanes 4, 5, 6, 7, and 8 in Fig. 2). The sample in lane 8 showed 3 different PCR bands produced by 3 sets of primers (Fig. 2). Because simplex RT-PCR appeared to be effective in the detection of a single virus in each individual sample, we tried to simultaneously detect multiple viruses present in lily samples via multiplex RT-PCR by using DPO primers (Table 1). In one reaction, multiplex RT-PCR was performed to investigate whether samples already confirmed to be infected with multiple viruses could simultaneously produce 3 virus-specific bands and another corresponding to the internal control. First, each DPO primer pair was evaluated to see if it could individually amplify a specific band in samples already confirmed to be infected with a virus (lanes 2–11 in Fig. 3). The amplified band in lane 1 (Fig. 3) represents 18S rRNA derived from a healthy sample, which was used as an internal control in this study. The multiplex RT-PCR results

Fig. 3. Multiplex RT-PCR detection of viruses in complex-virus infected lily samples. M, DNA size marker; Lane 1, healthy sample; 2, 3, and 4, virus-specific primers for corresponding samples infected with each virus; 5, 6, and 7, virus-specific and internal control primers for samples infected with each virus; 8, 9, and 10, primer pairs for duplex virus detection with internal control primers; 11, primer pairs for triplex virus detection with internal control primers. The arrows indicate the size of the RT-PCR product derived from the corresponding virus and 18S rRNA.

Fig. 4. Multiplex RT-PCR results for field-collected lily samples. M, DNA size marker; lanes 1–6, *Lilium* ‘Siberia’; lanes 7–11, *Lilium* ‘Sorbonne’; 12, *Lilium* ‘Valparaiso’; 13, *Lilium* ‘Donato’; +, positive control; –, negative control.

showed that bands of the expected sizes were amplified using the specific sequences of each target virus (Fig. 3). Lanes 5–7 demonstrate that 2 sets of primers amplified a single target viral gene and internal control gene (Fig. 3). Notably, when 3 sets of primers were used to amplify 2 different viral genes and an internal control gene, 3 different RT-PCR bands were produced (shown in lanes 8–10 of Fig. 3). Lane 8 shows that an RT-PCR product was not detected in lily samples infected with both CMV and LMoV. Therefore, we took the total RNA of each dried leaf sample that was previously confirmed to be infected with a virus and mixed the samples. All 4 sets of primers were then used to amplify cDNA corresponding to each virus in the sample, resulting in the amplification of 4 different sized amplicons. These results demonstrate that multiplex RT-PCR can be used to confirm triple infection in the lily plant, as shown in Fig. 2 and Fig. 3. In order to evaluate the multiplex detection system developed in this study, multiplex RT-PCR was applied to symptomatic field-collected samples. Various types of lily plants, such as the *Lilium* Oriental hybrid cultivars Siberia and Sorbonne as well as other cultivars, including Valparaiso and Donato, were used. The RT-PCR products of 3 different viruses (LMoV, CMV and LSV) were detected singularly in samples using multiplex RT-PCR as shown in lanes 1, 2, 3, 7 and 10 (Fig. 4). The double infection of LMoV and LSV was detected in lanes 4, 8 and 13, whereas CMV and LSV were detected together in lanes 5 and 9 via multiplex RT-PCR (Fig. 4). The fragments of 350, 285 and 205 bp, represent the cDNA of LMoV, CMV, and LSV, respectively, that were amplified from field samples along with an internal control fragment shown in lanes 6, 11 and 12 (Fig. 4). Each fragment was amplified as confirmed by comparing the PCR bands to those of the positive control that was previously detected by conventional RT-PCR. Three different viruses were detected showing correct size of virus-specific RT-PCR amplicons. Lanes 1 to 6 correspond to ‘Siberia’ lily samples that were collected in field. Based on Fig. 4, these samples were infected with single or multiple viruses according to multiplex RT-PCR. Field samples from ‘Sorbonne’ were also subjected to multiplex RT-PCR (Fig. 4, lanes 7–11) and

simultaneously analyzed for infection with single or multiple viruses. When samples collected from ‘Valparaiso’ and ‘Donato’ were evaluated, 2 and 3 viruses, respectively, were determined to infect these plants (Fig. 4, lanes 12–13). The multiplex RT-PCR results indicate that 2–3 viruses can be detected in various virus-infected lily cultivars. In addition, no significant primer interactions or primer-dimer formation were observed.

To simultaneously detect viruses in cultivated lily plants, a molecular detection method was developed and applied to field samples of lily plants. Each of the following viruses has been detected in lily plants with single, double, or triple infections (Figs. 1, 4). A previous study also detected the 3 different viruses above in the Oriental lily ‘Casa Blanca’ by using individual RT-PCR with degenerate primers (Niimi et al., 2003), and LSV and LMoV were found to coinfect ‘Sorbonne’ using an individual RT-PCR detection (Zheng et al., 2003). However, none of these studies have simultaneously detected multiple viruses infecting lily plants. Because the symptoms in lily plants infected with LSV differ in severity based on the sensitivity of the cultivar, growth conditions, as well as the susceptibility and sensitivity of the cultivar (Asjes, 2000), a more accurate and reliable detection system is needed. Moreover, the viral concentration depends on the growth stage of plant and varies depending on the plant organ and tissue (Kim et al., 1995). Thus, a reliable method of detection that is not influenced by different concentrations of viruses in plant tissue is required. Furthermore, plants are often infected by 2 or 3 different viruses in the field (Niimi et al., 2003). As a result, we developed an efficient detection system for screening multiple virus infections in lily plants using multiplex RT-PCR. Random hexamers were used to synthesize cDNA in a RT reaction for use in multiplex PCR. Random primers were designed and determined to be efficient for cDNA synthesis, which is in accordance with a previous report demonstrating that the cDNA from banana-infecting viruses was efficiently amplified using random primers compared to virus-specific or oligo dT primers (Liu et al., 2012). The authors also concluded that the primers used should anneal to sequences covering a wide range of isolates during multi-

plex PCR for the specific amplification of target viruses. The concentrations of primers used to amplify the cDNA of viruses and internal controls were optimized to determine the concentrations that result in the amplification of fragments from multiple viral genes. We initially adjusted the concentration of each primer to 10 pmol to perform simplex and multiplex RT-PCR. However, in the multiplex system, the smallest amplicon (205 bp) exhibited a weaker band on the gel than the larger fragments (data not shown). Therefore, we adjusted the primer concentration to 50 pmol to amplify the LSV-specific 205-bp fragment in multiplex PCR reactions, resulting in all amplicons having similar intensities on the gel (Fig. 3). Compared to previous report in which 200 ng of primer was used for PCR-based detection of virus infected lily plants (Sharma et al., 2005), the concentrations of primers and total RNA used in this study were significantly lower. Therefore, this method appears to be very efficient, especially for the detection of pathogens, such as virus, because their sequences are highly variable and have limited primer sites. In this study, we aimed to amplify relatively small PCR fragments because relatively large sizes over 500 bp could react with other primer pairs or long DNA fragments as reported in the study on detecting banana-infecting viruses (Liu et al., 2012). False-negative PCR results can be obtained by amplifying endogenous pararetrovirus cDNA in plant genomes, making it difficult to definitely detect virus infections (Geering et al., 2005). However, with the exception of the internal control, healthy lily samples lacked cDNA amplification in this study. A notable variation in band intensity was previously reported in bananas infected with multiple viruses when equal concentrations of primers were used in multiplex RT-PCR due to the different amplification efficiency of primer pairs under a variety of PCR conditions (Liu et al., 2012). The authors also did not detect 3 different viruses in banana. Therefore, compared to study on the detection of viruses infecting bananas, we observed similar intensities of DNA amplicons corresponding to 3 different viruses amplified by using DPO primers in multiplex RT-PCR as shown in Fig. 3. Multiplex PCR is a rapid and economical tool, but when multiple genes are amplified with corresponding primer pairs, primers often produce false-positives due to interactions between primers as well as different melting temperatures (Wei et al., 2008). To evaluate the efficiency of the multiplex system, we screened various lily samples and compared the RT-PCR amplicons with those of positive control reactions. Based on our results, multiplex RT-PCR using DPO primers performs highly specific cDNA amplification. The simultaneous detection of viruses could be useful for screening imported or exported lily bulbs for virus infections before or during planting and cultivation. According to a report on the infection rates of LMoV in

'Siberia', viral infections could potentially be prevented by identifying infected bulbs to reduce the spread of viruses during cultivation, decreasing the rate of infection during culture periods in the field (Kong et al., 2009). An unknown virion resembling closterovirus was detected in *Lilium* 'Casa Blanca' by electron microscopy analysis (Park et al., 2003). Therefore, it would be useful to develop a multiplex system that detects common lily-infecting viruses such as CMV, LMoV, and LSV, in addition to other lily-infecting viruses, such as LVX, TRV, TRSV and PIAMV. The lily plant is not a suitable system for experimentation for several reasons, including the observation that re-infection by lily infecting CMV is rare in lily host samples. Chen et al. (2001) mechanically inoculated lily plants with 13 CMV isolates, and only some of the CMV isolates originating from the lily plant could re-infect lily plants successfully. Several plants that were tested for a range of symptoms were found to be uninfected via sap inoculation from infected lily plants (Sharma et al., 2005). In addition, inoculation tests are not practical for LSV detection because they lack a local lesion assay host (Choi and Ryu, 2003). Precise virus detection by multiplex RT-PCR could be used to detect a variety of viral diseases. A study on quadruplex PCR detection of viral disease in *Musa* highlighted that virus-free planting material is very important in disease control because there is no effective resistance known in *Musa* (Liu et al., 2012). Likewise none of the lily plants are known to be resistant to viruses. The low incidence of virus infection in the initial virus-tested stocks obtained by select tissue culture procedures can lead to decreases in the access of vectors to virus-infected lily plants, according to Asjes's report (2000). High selection pressure for maintaining virus-free bulbs or bulblets via efficient screening methods, such as the system developed in this study, can increase healthy stock production. Our results may be useful for developing other multiplexing system for virus detection in lily plants. Moreover, this assay can be used for many purposes, especially for preventing the dispersal of contaminated bulbs through trade and for the purposes of identifying viruses for quarantine. This system can precisely detect multiple viruses in lily plants and yield equal target amplification with specifically designed primers and optimized reaction conditions. The development of rapid propagation using tissue culture as well as efficient and early screening via multiplex RT-PCR can facilitate virus-free cultivation for high quality lily propagation.

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