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LETTER TO THE EDITOR

Development of a real time RT-PCR with SYBR Green dye for the detection of several viruses from the bulbs and leaves of lily

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Lily plants can be infected by several viruses including Lily symptomless virus (LSV, the family *Flexiviridae*), Lily mottle virus (LMoV, the family *Potyviridae*), and Cucumber mosaic virus (CMV, the family *Bromoviridae*). All of them are found in Korea (*6, 12, 13*). In the field, these viruses can occur as a single or mixed infection within lily plants. Symptoms of the affected lily plants may vary from vein clearing, leaf mottle, leaf mosaic, chlorotic and yellow streaking, leaf curling, and brownish-necrotic spots. The efficient removal of diseased plants can prevent a development of mild symptoms in many cultivars, what may lead to an unacceptably high incidence of the virus in bulbs (*2*). Although ELISA is usually used to detect a single virus, it is not straightforward to detect a mixed infection by this method. In addition, high concentration of the virus can be reliably detected by ELISA, but this technique is not sensitive enough for the detection of a low virus concentration (*14*). Therefore, a more sensitive, simple, and efficient method for the detection of multiple infection should be developed. LMoV has a linear single-stranded positive-sense RNA genome of 9.6 kb that contains a single predicted ORF (*6*). The LSV genome contains a monopartite positive-sense RNA of 8.4 kb (*23*). The genome of CMV consists of 3 linear, positive-sense single-stranded RNAs with 5'-terminal cap structures (*5*). In previous studies, a specific amplification of target nucleic acid sequences by RT-PCR was widely used for the identification and detection of lily viruses (*9, 13*). Although conventional RT-PCR methods are more sensitive than the common ELISA assays, they are prone to a contamination. Recently, real time RT-PCR has been used for the detection and quantification of plant viruses (*3, 4, 7, 8*).

The objective of this study was to design a sensitive SYBR Green real time RT-PCR technique for the detection of LSV, LMoV, and CMV to cut down initial virus incidence in the lily supply.

Leaf and bulb samples showing characteristic symptoms of the virus infection were collected in different regions of Korea (provinces Gangwon, Chungnam, and Jeju) in the years 2008–2009. Standard samples known to be infected with LSV, LMoV, and CMV were obtained from the National Horticultural Research Institute (Suwon, Korea), stored at -80°C, and used as positive controls. Conventional RT-PCR for the specific detection of LSV, LMoV, and CMV was conducted using the previously described primers (*13*). The primers for real time RT-PCR were as follows: LSV-178 F 5'-ATGGTCATCATGTGTGCTTGCGTG-3', LSV-178 R 5'-GTACAAGCATGCTGTTCCACACGA-3', LMoV-171 F 5'-AGTCAACCTTGGGCA-CCTTGTGA-3', LMoV-171 R 5'-CAATGCACCAAACCATCAGACCGT-3', CMV-193 F 5'-AGCCTCACCGGTACTGGTTTATCA-3', CMV-193 R 5'-AATGCGTTGGTGCTCGATGTCAAC-3'. Real time RT-PCR was conducted using a PrimeScript II 1st strand cDNA synthesis kit and a SYBR premix Ex Taq II kit (Takara). Standard curves were generated from the

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Abbreviations: CMV = Cucumber mosaic virus; LMoV = Lily mottle virus; LSV = Lily symptomless virus

10-fold serial dilutions of recombinant plasmids containing the RT-PCR product of the coat protein gene. The plasmids were purified and their concentrations were determined by measuring A_{260}. A corresponding copy number was then calculated according to the previously described formula (*22*). C_T (threshold) values in each dilution were measured using real time PCR to generate standard curves for LSV, LMoV, and CMV. The standard curve was used to calculate a relative quantification of particular lily virus cDNA in the field samples.

The entire coat protein gene was amplified from a collection of 31 field samples (12 leaf and 19 bulb samples) showing characteristic symptoms of the viral infection by one step RT-PCR using the previously described lily virus-specific primers. The results showed that 9 plants were infected with LSV, 14 plants with LMoV, and 1 plant with CMV. Real time RT-PCR primers were designed from the same region that was amplified using conventional RT-PCR primers. To construct a reference plasmid containing the target gene, a conventional RT-PCR fragment much larger than the amplicon produced by real-time RT-PCR was used for the cloning. The use of cloned target allowed evaluation of absolute viral cDNA copy number by the use of standard curve. Standard curves based on the threshold cycles (C_T) for 10-fold serial dilutions of the recombinant plasmids were constructed and then the dilutions from 1×10^8 to 1×10^0 were amplified by the real time PCR. Stable amplification could be observed for as few as 100 copies of the plasmids (LSV and LMoV). However, when the amount of plasmid copies was reduced to the number below 100, no stable amplification could be obtained. Quantification of the LSV in field samples was performed by comparison of C_T value of each sample to the C_T values of standard regression lines. Under these conditions, the number of cDNA/LSV copies ranged from 5×10^7(leaf) to 1×10^6 (bulb) per µl. Seventeen samples negative for the LSV by conventional RT-PCR were examined by the real time RT-PCR and 13 samples were found to be LSV-positive. Four samples were negative by both tests (Table 1). These results indicated that real time RT-PCR was more sensitive than the conventional RT-PCR.

Table 1. Comparison of RT-PCR and real time RT-PCR for the detection of LSV

Origin of isolates	Positive samples	
	RT-PCR	Real-time RT-PCR
Leaves (12[a])		
Chungnam (3)	–	–
Jeju (9)	2	7
Bulbs (19[b])		
Chungnam (9)	6	9
Gangwon (3)	–	2
Jeju (7)	1	4
No. of positive/total	9/31	22/31

[a,b]Total number of tested leaves and bulbs, respectively.

Quantitative real time PCR methods are based on the differently labeled specific probes that employ hydrolysis (TaqMan), which requires a high complementation in the probe-binding site (*1, 17, 18, 19*). In contrast, SYBR Green dye binds non-specifically to the double-stranded DNA by intercalation. Even though the TaqMan system is rapid, sensitive, sequence-specific, and has the potential for multiflexing, the SYBR Green system is simpler, cheaper, and able to detect non-described variants (*10, 11, 15, 16, 20, 21*). Based on the conserved regions within the coat protein gene from our isolates and reference viruses, specific primers for each viral sequence were designed. All primers effectively amplified the desired size. In addition, the predicted PCR product was confirmed by the melting curve analysis and agarose gel electrophoresis. Non-specific products or primer dimers were not observed. For the relative quantification of viral RNA copies, standard curves were generated using the recombinant plasmids for LSV, LMoV, and CMV. The lower detection limits of SYBR Green real time RT-PCR for LSV, LMoV, and CMV were 1.0×10^2, 1.0×10^2, and 1.0×10^3 viral copies/µl, respectively. The limit of detection was recorded as the largest dilution that provided a positive result. When we used LSV cDNAs from field isolates as a template, viral copy numbers ranging from 5×10^7 (leaf) to 1×10^6 (bulb) were obtained.

Comparison of SYBR Green real time RT-PCR to the conventional RT-PCR revealed that SYBR Green RT-PCR detected viruses in lily samples that were negative by the conventional RT-PCR. These findings indicated that the SYBR Green real time RT-PCR developed in this study was sensitive and specific enough to detect the LSV isolates from geographically diverse regions.

In conclusion, we developed a SYBR Green real time RT-PCR method suitable for the rapid, specific, robust, high throughput, and sensitive detection of lily viruses. The assay developed here could allow a large-scale lily virus testing for the bulb-certification and strict post-entry quarantine. Our method also allows a pre-symptomatic detection of lily viruses, which is important for the reduction of disease incidence by the early eradication of primary virus reservoirs in the field.

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