Development of an Indirect ELISA and Immunocapture RT-PCR for Lily Virus Detection

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Multiple viruses such as Lily symptomless virus (LSV), Lily mottle virus (LMoV), and cucumber mosaic virus (CMV) are the most prevalent viruses infecting lilies in Korea. Leaf samples and bulbs showing characteristic symptoms of virus infection were collected from Gangwon, Chungnam, and Jeju provinces of Korea in 2008–2011. Coat protein (CP) genes of LSV and LMoV were amplified from collected samples by reverse transcription-polymerase chain reaction (RT-PCR) and cloned into a pET21d(+) expression vector to generate recombinant CPs. The resulting carboxy-terminal His-tagged CPs were expressed in Escherichia coli strain BL21(DE3) by isopropyl-1-thio-β-D-galactoside induction. The recombinant proteins were purified using Ni-NTA agarose beads, and the purified proteins were used as an immunogen to produce polyclonal antibodies in rabbits. The resulting polyclonal antisera recognized specifically LSV and LMoV from infected plant tissues in Western blotting assays. Indirect enzyme-linked immunosorbent assay and immunocapture RT-PCR using these polyclonal antisera were developed for the sensitive, efficient, economic, and rapid detection of Lily viruses. These results suggest that large-scale bulb tests and economic detection of Lily viruses in epidemiological studies can be performed routinely using these polyclonal antisera.

Keywords: Coat protein, IC-RT-PCR, ID-ELISA, polyclonal antisera
an earlier study, specific amplification of target nucleic acid sequences using reverse transcription-polymerase chain reaction (RT-PCR) was used widely for the identification and detection of Lily viruses [7, 11]. Although conventional RT-PCR methods are more sensitive than common ELISA assays, they are prone to contamination and RNA extraction is troublesome. Inhibitors present in plant constituents can also limit the sensitivity of conventional RT-PCR. Real-time RT-PCR has been used recently for the detection and quantification of plant viruses [3, 4, 12]. Real-time RT-PCR allows for rapid target-specific amplicon detection, high-throughput virus detection, and accurate quantification without post-PCR processing. Although real-time RT-PCR is potentially very useful for the detection of plant viral infections, its application to Lily virus detection has been limited by the lack of trained personnel and the need for expensive equipment. Among these detection methods, ELISA is cost effective and suitable for the rapid detection of large-scale samples. ELISA is still an important diagnostic tool, and the preparation of a good qualitative antibody is important to achieve high sensitivity. Various ELISA alternatives based on polyclonal or monoclonal antibodies are used widely to improve detection limits and broaden strain specificity in plant virology [14, 16, 17].

Bacterial expression systems are stable, have high productivity, and offer easy purification. Therefore, the plant viral CPs expressed in these systems have been reported for several plant viruses [8, 15]. Use of this technique overcomes purification difficulties of virus preparations of virus-specific antisera. LSV and LMoV are very difficult to purify for the generation of antisera. LSV is a filamentous virus, so it aggregates upon ultracentrifugation. LMoV is distributed unevenly within the plant tissue, and it is difficult to obtain homogenous virus particles in mixed virus infections. Therefore, to overcome Lily virus purification difficulties, recombinant viral CPs expressed in bacterial systems have been used as immunogens.

In this study, LSV and LMoV CPs were expressed in Escherichia coli using the pET-21d(+) expression system, whereas polyclonal antibodies (PAbs) were produced using recombinant CPs. Indirect ELISA (ID-ELISA) and immunocapture RT-PCR (IC-RT-PCR) using the prepared polyclonal antibodies were developed and used for the detection of LSV and LMoV in field samples.

**RNA Extraction**
Infected leaves and bulbs were ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The tissues were added to a microcentrifuge tube containing 1 ml of Trizol reagent and 0.2 ml of chloroform. The homogenate was incubated at room temperature (RT) for 2–3 min and then centrifuged at 12,000 × g for 15 min at 2–8°C. Aliquots (500 µl each) from the aqueous phase were then transferred to a fresh tube and the RNA was precipitated for 10 min at 15–30°C by the addition of an equal volume of isopropyl alcohol. The sample was then centrifuged for 10 min at 2–4°C and washed with 1 ml of 75% ethanol. Finally, the pellet was resuspended in 25 µl of diethylpyrocarbonate-treated water.

**Cloning and Prokaryotic Expression of the CP Gene**
Primers for the specific detection of LSV and LMoV were designed based on the alignment of the CP sequence of each pathogen available in the GenBank database. The LSV CP1 forward primer (5'-CCATGGATCAAGACACGAC-3', Ncol restriction site is underlined), the LSV CP2 reverse primer (5'-CTCGAGTCTTGATTGCGTATCG-3', XhoI restriction site is underlined), the LMoV CP1 forward primer (5'-CCATGGCAAAAATGAGACCTTAAC-3', Ncol restriction site is underlined), and the LMoV CP2 reverse primer (5'-CTCGAGCTATGAAAATCCAGAAGT-3', XhoI restriction site is underlined) were used to amplify the CP gene of LSV and LMoV, respectively. Reverse transcription and subsequent PCR reactions were conducted using an MJ Research PTC-150 Thermal Cycler (PE Applied Biosystems, Carlsbad, CA, USA) with a Maxime RT-PCR PreMix kit (Intron Biotechnology, Korea). The thermal cycling scheme was 42°C for 45 min, followed by 30 cycles of 95°C for 30 s, 50°C for 1 min, and 72°C for 30 s, with a final extension at 72°C for 7 min. The PCR products were examined by electrophoresis in 1.5% agarose gels.

The RT-PCR products were cloned into a pGEM-T Easy Plasmid Vector (Promega, Madison, WI, USA) for construction of the recombinant plasmids. The target fragment was released from the cloned pGEM-T Easy Plasmid using Ncol/Xhol and inserted into the corresponding sites of the pET21d(+) expression vector (Novagen, Gibbstown, NJ, USA). The recombinant plasmid was transformed in E. coli strain BL21(DE3). Protein expression was induced at an absorbance of 0.3 at 600 nm using isopropyl-1-thio-β-D-galactoside (IPTG) in a final concentration of 1 mM and the culture was incubated with shaking at 37°C for 5 h. CP was extracted using lysis buffer containing 50 mM NaHPO₄, 300 mM NaCl, 30 mM imidazole (pH 8), 1 mg/ml lysozyme (Sigma-Aldrich, St. Louis, MO, USA), and protease inhibitor cocktail (Calbiochem, Gibbstown, NJ, USA) and then incubated on ice for 30 min. The culture was freeze-thawed twice and centrifuged at 10,000 × g for 30 min at 4°C. The supernatant containing 6× His-fused CP was purified using Ni-NTA agarose beads (Qiagen, Hilden, Germany). The protein-binding beads were loaded onto a column and washed twice with wash buffer [50 mM NaHPO₄, 300 mM NaCl, 50 mM imidazole (pH 8)]. The 6× His-fused CP was then eluted with elution buffer [50 mM NaHPO₄, 300 mM NaCl, and 300 mM imidazole (pH 8)]. The purified protein solution was quantified with respect to standardization with bovine serum albumin and estimated by its molar extinction coefficient at 595 nm. The purified recombinant CP was used as an immunogen and injected into rabbits.
Western Blot Analysis

Leaf samples from healthy or virus-infected plants were homogenized in sodium dodecyl sulfate (SDS) extraction buffer according to Laemmli [10]. The samples were denatured by boiling for 3 min and then separated on 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The proteins were then transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline Tween-20 (TBS-T) buffer containing 5% skim milk powder at 4°C overnight and incubated with PAbs specific to LSV and LMoV at RT for 2 h. After three washes in TBS-T, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (KOMAbiotech, Korea) at RT for 1 h. The blots were then visualized using 3,3-diaminobenzidine.

ID-ELISA

ID-ELISA was performed using polyclonal LSV and LMoV antisera. Healthy or virus-infected leaf samples were homogenized in extraction buffer and diluted. One hundred µl of each sample in the coating buffer was added to each well of a 96-well microplate and incubated overnight at 4°C. After three washes with 200 µl of phosphate-buffered saline with Tween-20 (PBS-T), 100 µl of diluted CP antiserum was added to each well and the microplate was incubated at 37°C for 1 h. After 1 h of incubation, the unbound compounds were removed by five washes with PBS-T. Goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP; 100 µl) was added and incubated for 1 h at 37°C. The absorbance value of the developed color was measured at 650 nm.

IC-RT-PCR

Leaf samples from Lily plants showing disease symptoms were homogenized in extraction buffer and diluted. Supernatants were kept on ice. Two hundred µl of leaf sap was centrifuged at 13,000 rpm for 1 min and the supernatants were kept on ice. Two hundred µl of each sample in the coating buffer was added to each well of a 96-well microplate and incubated overnight at 4°C. After three washes with 200 µl of phosphate-buffered saline with Tween-20 (PBS-T), 100 µl of diluted CP antiserum was added to each well and the microplate was incubated at 37°C for 1 h. After 1 h of incubation, the unbound compounds were removed by five washes with PBS-T. Goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP; 100 µl) was added and incubated for 1 h at 37°C. The absorbance value of the developed color was measured at 650 nm.

RESULTS

Cloning and Expression of LSV and LMoV CPs

LSV CP (876 bp) and LMoV CP (825 bp) were cloned from genomic RNA extracted from virus-infected Lily by RT-PCR, and the sequence was confirmed using an automated DNA sequencing system (LSV, GenBank Accession No. JN 830615; LMoV, GenBank Accession No. JN 830617). The amplified fragments were inserted between the NcoI and XhoI sites in pET-21d(+) to produce a C-terminal His-tagged CP fusion. The resulting plasmids were designated pET-21d(+)LSV-CP and pET-21d(+)LMoV-CP, respectively. SDS-PAGE revealed that the recombinant LSV CP band with a molecular mass of 32 kDa was expressed successfully in IPTG-induced pET-21d(+)LSV-CP–transformed bacteria but not in pET-21d(+)transformed control bacteria (Fig. 1A). Recombinant His-tagged LSV-CP and LMoV-CP were purified from the soluble bacteria fractions through Ni-NTA agarose chromatography. (A) Lane M: protein marker; Lane 1: extracts from Escherichia coli BL21 transformed with the pET-21d(+) plasmid; Lane 2: protein products of bacteria transformed with pET-21d(+)–Lily symptomless virus (LSV) coat proteins (CPs) after 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) at 37°C. (B) Lane M: protein marker; Lane 1: protein products of bacteria transformed with pET-21d(+)–LMoV CP after IPTG induction eluted from the Ni-NTA affinity column. (C) Lane M: protein marker; Lane 1: protein products of bacteria transformed with pET-21d(+)–Lily mottled virus (LMoV) CP after IPTG induction eluted from the Ni-NTA affinity column.

Detection of LSV and LMoV Using Polyclonal Antibodies by Western Blotting

To evaluate the suitability of PAbs to LSV, antibodies recovered from rabbits were diluted subsequently to 1:10,000 for Western blotting. Fig. 2 shows that the antibody could react with LSV-infected plant tissue extracts and the recombinant His-LSV CP, but not with the healthy samples. Purified recombinant His-LMoV CP and LMoV-infected Lily plants were used to evaluate LMoV-specific PABs, which bound specifically to LMoV in the infected Lily samples and recombinant His-LMoV, but not the healthy samples (Fig. 3).

ID-ELISA

Rabbit PABs against the recombinant LSV CP and LMoV CP were used in the ID-ELISA. To determine the suitable
dilution of PAb, various dilutions of antisera (1:5,000, 1:10,000, 1:20,000, and 1:30,000) were tested using leaf saps from virus-infected or healthy lilies. A total of 24 samples were collected from the *Lilium* Oriental hybrid ‘Yelloween’ and ‘Siberia’ showing mosaic symptoms in the Taean and Gangneung provinces of Korea in 2011. The results of three repeats suggested that the dilution of LSV PAb at 1:20,000 and LMoV PAb at 1:30,000 worked well in ID-ELISA. Our established ID-ELISA method could be used to detect infections in leaf saps (Fig. 4).

**IC-RT-PCR for Detection of LSV and LMoV**

The IC-RT-PCR method was used to test for the presence of LSV and LMoV in field samples. PAb (100 µl) diluted at 1:100 in coating buffer was used to coat the PCR tube and 100 µl of leaf sap was used for detection. LSV primers produced a single 655 bp faint band from Lily samples showing mosaic symptoms, which were collected from *Lilium* Oriental hybrid ‘Yelloween’ and ‘Siberia’ showing mosaic symptoms. Negative controls were wells incubated with leaf extracts from healthy leaf (sample 1).

**DISCUSSION**

The diversity of symptoms caused by LMoV, LSV, and CMV prevent the easy selection and removal of diseased
Lily plants in the field. Therefore a simple, specific, and sensitive technique that is capable of detecting very low numbers of virus particles is needed. The use of real-time RT-PCR in the field of plant virus diagnosis has increased greatly because it uses reduced cycle times and does not require post-PCR analysis. Virus quantification is also possible by real-time RT-PCR, a method that has been applied successfully to the detection of other plant viruses [3, 4, 13]. Although both conventional and real-time RT-PCR have been used for the rapid detection of lily viruses, this method is not suitable for large numbers of samples.

In contrast, ELISA is a cost-effective and suitable method for the rapid detection of large-scale samples. To achieve high specificity and sensitivity of ELISA, the preparation of a good qualitative antibody is important. However, owing to the fragility and uneven distribution of LMoV, it is difficult to purify this virus to prepare antiserum. In addition, contamination problems are possible during virus purification because lilies are often infected simultaneously by two or three different viruses in the field. The use of recombinant CP expression in prokaryotic systems overcomes this problem because it is simple, fast, abundant, inexpensive, and easy [2, 8, 15].

In this study, the LSV CP and LMoV CP were expressed in E. coli, and two PAbs were produced in rabbits. A single protein band with a molecular mass close to those of LSV and LMoV was specifically detected by Western blotting in virus-infected leaf samples (Fig. 2 and 3). These findings indicate that the PAbs prepared in this study are highly specific for LSV and LMoV, respectively. ID-ELISA methods based on PAbs has been established for the efficient detection of LSV and LMoV. LMoV PAbs at dilutions of 1:30,000 reacted more strongly than LSV PAbs at dilutions of 1:20,000; however, both enabled detection of LSV and LMoV in field lily samples.

We also developed an IC-RT-PCR procedure that offers sensitive, specific, and rapid detection of LSV and LMoV from field samples. This method avoided RNA extraction and was carried out easily in a single tube. IC-RT-PCR methods have already been developed for detecting many other plant viruses [16]. Using the IC-RT-PCR assay, we found that a single 651 bp band could be amplified from LMoV-infected leaf sap, whereas no DNA band was amplified from healthy leaf sap. However, LSV primers produced a faint band from Liliy samples showing mosaic symptoms, which were collected from Lilium Oriental hybrid ‘Siberia’ in different regions. These results suggested that the concentration of LSV in field samples is lower than that of LMoV. In addition, our IC-RT-PCR procedure needs to be improved to increase the sensitivity of the IC-RT-PCR for LSV detection. Fig. 5 shows that IC-RT-PCR detected LMoV in some Lilium samples that conventional RT-PCR did not. These findings indicate that the sensitivity of the IC-RT-PCR method developed in this study is relatively higher than that of conventional RT-PCR.

In conclusion, LSV CP and LMoV CP were expressed in E. coli and two PAbs were produced. Using these PAbs, ID-ELISA and IC-RT-PCR were established for the detection of Lily viruses in field samples. These two methods can be used for virus surveillance to help reduce economic losses in the Lily industry.

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References


