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Purification and immuno-gold labeling of lily mottle virus from lily leaves

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Lily mottle virus (LMoV) is prevalent in *Lilium* species worldwide causing dwarfing, flower breaking, and reduced bulb yield. In this paper, an easy to use and efficient procedure is described for purification of LMoV from lily leaves. The resulting sample is characterized by a 260/280 nm absorbance ratio of 1.20 at a concentration of 1.27 mg/ml. The procedure results in high protein purity and particle integrity as shown by UV-spectrophotometry, polyacrylamide gel electrophoresis (PAGE), Western blotting, reverse transcriptase (RT)-PCR and transmission electron microscopy (TEM) in combination with immuno-gold labeling. This is the first time that an immuno-gold labeling (IGL) assay was performed to identify a virus of lily. Purified products can be used as a source of antigen in the preparation of antibodies against LMoV and may assist in the development of a diagnostic test for LMoV and in epidemiological surveys.

1. Introduction

Viral diseases cause quantitative and qualitative yield reductions of lilies around the world (Wang et al., 2010). More than 10 different viruses have been reported to infect lilies worldwide, and lily mottle virus (LMoV) is one of the most common (Zhang et al., 2014). LMoV is closely related to the tulip breaking virus (TBV; Alper et al., 1982) and is a member of the *Potyvirus* genus within the Potyviridae family. It is aphid transmitted and only infects plants of the genera *Lilium* and *Tulipa* L. (*Liliaceae* family) (Derks et al., 1994). The particles of LMoV are flexuous, non-enveloped, and rod-shaped; they are 680–900 nm long and 11–15 nm wide (King et al., 2011). Symptoms of LMoV in lily include leaf mottle, leaf mosaic, reddish-brownish necrotic spots, vein clearing, chlorosis and yellow streaking, and leaf curling and narrowing (Fig. 1). Other symptoms included dwarfing, flower breaking and reduced bulb yield. The symptoms may be more severe if plants are simultaneously infected with Cucumber mosaic virus (CMV) and Lily symptomless virus (LSV) (Asjes, 2000; Zhang et al., 2014). Symptoms may also be very mild, or plants maybe symptomless during early growth stages.

Enzyme-linked immunosorbent assay (ELISA; Sharma et al., 2005; Tong et al., 2010) is commonly used to detect LMoV in lily. However, the method is time consuming, and requires technical expertise and specialized laboratory equipment. Therefore, an immunochromatographic strip (ICS) test was developed to rapidly detect LMoV (Zhang et al., 2015). The ICS assay can be completed within 10 min without the need for special instruments or skilled personnel, and can be used virtually anywhere. Currently, immunochromatographic strips are prepared using two anti-LMoV polyclonal antibodies, to capture and detect LMoV particles (Zhang et al., 2015). Polyclonal antibodies are prone to batch-to-batch variability, which could affect the stability of the ICS test. In contrast, homogeneity of monoclonal antibodies is very high. Once hybridomas are made, a constant and renewable source of antibodies would be available, enabling improved standardization of the ICS assay.

Simple and easy to use methods have been published for the purification of LSV and CMV from lily (Hsu et al., 1995; Wang et al., 2007; Liang et al., 2009), and purification of potyviruses from host plants has been described (Rupar et al., 2013). There is no information however, on the purification of LMoV from lily leaves. Purification of viral particles of LMoV is an important step in the production of highly specific antibodies and thus there is a need for more research. In this study, an easy to use and efficient

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Fig.1. Lily leaves exhibiting mottle symptoms.

procedure for isolation and purification of LMoV particles was developed. The viral protein purity and morphological intactness of purified viral particles were verified by SDS-PAGE, Western blotting, RT-PCR and transmission electron microscope. In addition, we report the successful application of an immuno-gold labeling (IGL) assay to detect and unequivocally identify LMoV particles. To our knowledge, this is the first time that the IGL technique was applied to the identification of a lily virus.

2. Materials and method

2.1. Viral material

We collected naturally infected plants of oriental hybrid lily (*Lilium oriental* cv. Sorbonne) showing visual dwarfing, or leaves displaying chlorotic yellow spots, stripes or mosaic symptoms at the Gaolan Research Station (Gaolan County, Lanzhou, Gansu Province, China, 36°13′N 103°47′E).[1] Leaves near the flower bud were tested by RT-PCR according to the method of Zhang et al. (2010). Leaves from plants that tested positive for LMoV served as sources of LMoV and were stored at −70 °C.

2.2. Purification of LMoV

Twenty grams of LMoV positive leaf tissue was crushed into powder in liquid nitrogen with a mortar, homogenized quickly with chilled (4 °C) extraction buffer (EB: 0.5 M potassium phosphate (pH7.0) containing 10 mM EDTA and 0.1% (v/v) 2-mercaptoethanol) in a 1:4 (w:v) ratio. The resulting crude homogenate was passed through a cloth and centrifuged at 5000 × *g* for 5 min at 4 °C to remove plant debris. Chloroform was added to the supernatant to a concentration of 50% (v/v) and intensely stirred for 15 min and then centrifuged at 5000 × *g* for 15 min. First PEG-6000 and then NaCl was slowly added to the supernatant to concentrations of 7.5% and 4% w/v, respectively. The mixture was kept at 4 °C and stirred slowly for 30 min and then centrifuged at 8000 × *g* for 20 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 20 ml of resuspension buffer (RB: 0.5 M potassium phosphate (pH 7.0) containing 10 mM EDTA, 0.1% (v/v) 2-mercaptoethanol and 2% Triton X-100) and stored overnight. The second day the suspension was centrifuged at 10000 × *g* for 20 min. First PEG-6000 and then NaCl were slowly added to the supernatant to concentrations of 6% and 3% w/v, respectively. The mixture was kept at 4 °C and stirred slowly for 20 min and centrifuged at 8000 × *g* for 20 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 20 ml of RB. The suspension was kept at 4 °C with slow stirring for 20 min and centrifuged at 10000 × *g* for 20 min at 4 °C. Subsequently, the suspension was ultracentrifuged at 25,000 × *g* for two and a half hours at 4 °C. The supernatant was discarded; the pellet was resuspended in 500 μl EB and stirred slowly at 4 °C for 1 h and then centrifuged at 5000 × *g* for 10 min at 4 °C to obtain the final suspension of pure LMoV for further analysis.

2.3. Preparationof LMoV antiserum

The rabbit anti-LMoV antiserum was obtained following the procedure of Tong et al. (2010). Briefly, the coat protein (CP) gene of LMoV was amplified by RT-PCR from infected plants of oriental hybrid lily (*L. oriental* cv. Sorbonne) and cloned into the prokaryotic pET-28a vector to generate the recombinant plasmid pET-28a-CP. The resulting carboxyterminal His-tagged CP was over-expressed in *Escherichia coli* BL 21 cells by isopropyl-b-D-thiogalactoside (IPTG) induction and purified over Ni-NTA affinity columns. The purified CP was used to produce polyclonal antiserum in rabbits. Rabbit anti-LMoV CP IgG was purified by ammonium sulfate graded precipitation followed by DE52 anion-exchange chromatography.

2.4. Virus purity estimation by Abs 260/280

Concentration of the pure viral suspension was determined by measuring the UV-absorbance at 260 nm and 280 nm with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The ABS_{260}/ABS_{280} ratios of viral products were calculated to determine the purity.

2.5. SDS-PAGE and Western blotting analysis

Purified virus samples were mixed with 2 × SDS-PAGE loading buffer, and then boiled for 5 min. SDS-PAGE was carried out on a 12% acrylamide gel. Protein bands were visualized by Coomassie Blue R250 staining. For Western blotting, the proteins were separated by 12% SDS-PAGE under a reducing condition and then electroblotted onto a nitrocellulose membrane (Millipore, USA) using a Semi-Dry Transfer System (Bio-Rad, USA). Non-specific protein binding was blocked by incubating the membrane overnight at 4 °C in TBS containing 0.05% Tween-20 and 5% skim milk. After washing with TBS-Tween 20 (TBST), the membrane was incubated with a 1/3000 dilution of rabbit anti-LMoV CP IgG (at 2.1×10^{-3} mg/ml) at 37 °C for 1 h under constant shaking. The membrane was washed five times with TBST and incubated at 37 °C with the appropriate Alkaline phosphatase AffiniPure goat anti-rabbit IgG for 1 h, and subsequently washed five times with TBST. Conjugates binding to the membrane were visualized using nitrobluetetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) as the substrate.

2.6. Nucleic acid extraction and RT-PCR analysis

The viral RNA of LMoV preparations was extracted from 100 μl samples using RNAprep pure Plant Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. Final elution was done with 50 μl of RNAse free H_2O. The RNA concentration of the sample was measured with a NanoDropND-1000 spectrophotometer (NanoDrop Technologies,Wilmington, DE, USA). Subsequently, a quadruplex RT-PCR assay was used to detect the viral RNA (Zhang et al., 2010, 2015). Due to its abundance and uniform distribution in plant cells, the 18S rRNA housekeeping gene is usually used as an internal control (Zhang et al., 2015). The first strand of cDNA was synthesized by M-MLV reverse transcriptase with the Oligo(dT)-18 primer (Takara Biotech, Dalian, China). Quadruplex PCR was carried out in 25 μl volumes containing 2 μl

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