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Production and Evaluation of Monoclonal Antibodies Against Recombinant Coat Protein of *Lily mottle virus* for Western Blotting and Immono-blot Analysis

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***Lily mottle virus* (LMoV) causes flower quality reduction in *Lilium* spp. The coat protein gene was RT-PCR-amplified from total RNA extracted from infected lily leaves and the amplified fragment was cloned into the pRSET expression vector tagged with a His-MBP. The plasmid of recombinant coat protein was used to transform an *Escherichia coli* strain pLysS and was expressed. The coat protein was purified by affinity chromatography using a Ni-NTA resin. The identity of the purified protein was confirmed by SDS-PAGE. The *in vitro*-expressed protein was used for immunization of mice. The polyclonal and monoclonal antibodies reacted specifically for the detection of LMoV in lily extracts in Western blot. Moreover the monoclonal antibodies reacted with lily extracts in DAS-ELISA with no unspecific or heterologous reactions against other non-serologically related viruses, but the polyclonal antibodies revealed a weak reaction against both infected lily and healthy control.**

Keywords : Lily mottle virus, monoclonal antibody coat protein, lily

Several viruses have been reported to occur in lily, often as mixed infections, reducing their vigour and marketability (Bellardi et al., 2002; Chung et al., 2003). These include *Lily mottle virus* (LMoV), *Cucumber mosaic virus* (CMV), *Lily symptomeless virus* (LSV) and *Lily virus* X (LVX) (Allen, 1972; Jung et al., 2000; Shukla and Ward; Van Slogteren, 1971). LMoV is an aphid borne virus, occurs in lily growing regions of the world including Korea (Chung et al., 2003), China (Zheng et al., 2003), Italy (Bellardi et al., 2002) and The Netherlands (Dekker et al., 1993). LMoV causes leaf mosaic and flower breaking symptoms (Dekker et al., 1993; Van Slogteren, 1971).

Virus purification is usually a labor-intensive procedure with occasionally unsatisfactory results concerning purity and concentration of final preparation. In case of LMoV, the lack of adequate herbaceous hosts is a main difficulty to purify virus particles.

With the development of molecular biology techniques, cloning and expression of viral genes coding for structural and non-structural proteins has become an important strategy for obtaining large amounts of antigens with stable properties (Barbieri et al., 2004; Čeřovská et al., 2003; Fajardo et al., 2007; Ling et al., 2000; Minafra et al., 2000).

The main objective of this study was to produce and characterize specific antibodies against the recombinant coat protein of LMoV isolate LMoVK-LiCB and evaluate them for diagnostics. The monoclonal antibodies (MAbs) and poly clonal antibodies (PAbs) raised against LMoVK-LiCB-CP were compared in DAS-ELISA.

Materials and Methods

Reverse transcription and polymerase chain reaction (RT-PCR) of coat protein (CP) of LMoV. The CP gene of LMoV, isolate LMoVK-LiCB, was amplified by RT-PCR with a primer pair. Sense primer LMOV-F contained *Bam*HI restriction site (5-ACTGGATCCGCAAATGAGA-CACTC-3) and antisense primer LMOV-R contained *Xho*I restriction site (5-GTCACTCGAGCTACATAGAAATTC-CAAG-3). The reaction was carried out in 30 cycles of 30 second denaturation at 94 °C, 30 second annealing at 55 °C and 1 min elongation at 72 °C and 10 min additional extension at 72 °C.

Cloning of the CP gene of the LMoVK-LiCB. RT-PCR products were digested with *Bam*HI and *Xho*I and ligated to the *Bam*HI and *Xho*I-digested expression vector His-MBPpRSET (Invitrogen, USA) and cloned in *E. coli* Top10. One recombinant clone was designated as pRSET-LMOVK-LiCB in this study. Amino acid sequences of pRSET-LMOVK-LiCB are indicated in Fig. 1 and under lined letters are His-MBP region.

Expression of CP gene. For in vitro expression, the construct pRSET-LMOVK-LiCB was transferred to an *E. coli* strain pLysS by a heat shock procedure. One colony

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was incubated and cultured in 5 ml of LB/ampcillin at 37°C and the seed culture was added to 1 liter of LB/amp. The culture was grown until an optical density at 600 nm (OD_{600}) of 0.5 and then induced with IPTG in a final concentration of 0.5 mM. The culture was then incubated for 3 h at 25°C.

Preparation of CP-enriched fractions. Cells were harvested by centrifugation at 4500 rpm for 10 min and were resuspended in 200 ml of buffer A (20 mM Tris pH 8.0, 0.5M NaCl, 5 mM imidazole) followed by sonication. Supernatant was collected by centrifugation at 10,000 rpm for 15 min, and was applied to Ni-NTA affinity columns (Qiagen, USA) according to the manufacturer's instructions, followed by washing with buffer A. Target protein was eluted using buffer B (20 mM Tris pH 8.0, 0.5 M NaCl, 250 mM imidazole), and fraction containing desired protein was collected and dialyzed against 20 mM Tris buffer, pH 8.0. Protein was concentrated using 10,000 MWCO, and 3 μl of concentrated protein loaded in SDS-PAGE. Protein quantified with the Bradford reagent (Bio-Rad, USA) according to the manufacturer's instructions.

Production of PAbs. Five 6-week-old female BALB/c mice were immunized subcutaneously with the purified 100 μg of LMoVK-LiCB-CP recombinant protein emulsified with Freund's complete adjuvant. Subsequently two booster injections were given at 2-week intervals with 50 μg of recombinant protein subcutaneously, in Freund's incomplete adjuvant. Blood gathering was conducted after 1st injection by heart puncture. Immunoglobulins were purified from the PAbs by affinity chromatography with protein A column (Pall life Sciences, USA), according to the manufacturer's instructions.

Production of MAbs. Five six-week-old BALB/c female mice were immunized by one intraperitoneal injection of 100 μg of recombinant LMoVK-LiCB-CP emulsified with Freund's complete adjuvant. Their spleens were removed aseptically after three booster injections given at 2 week intervals. Spleen cells were mixed with the myeloma cell line F0 obtained from American Type Culture Collection (ATCC), cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). Hybridoma lines that produced antibodies to LMoV CP were subcloned twice by the limiting dilution technique and selected by indirect ELISA assay (Harlow and Lane, 1988).

Ascitic fluid was produced as described by Harlow and Lane (1988). Female BALB/c mice were injected with 0.5 ml of pristane (Sigma, St. Louis, USA) into the peritoneum. After 7-14 days, 5×10^6 hybridoma cells in 0.5 ml PBS buffer (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl pH 7.4) per 1 mouse were injected. Ascitic fluid was collected within 14 days following the injection of the cells by a needle attached to a 5 ml syringe. Immunoglobulins were purified from MAbs by affinity chromatography with Protein G Sepharose 4 Fast Flow (GE healthcare, USA) following the manufacturer's protocol.

Immunosorbent electron microscopy (ISEM). To illustrate the specificity of the MAbs produced we performed an immune decoration of virus particles from LMoV and serologically unrelated *Potato virus* Y (PVY). We followed the procedure described by Milne and Luisoni (1977) with modifications. A carbon-coated grid was floated on 1:1000 diluted antibody for 5 min at room temperature. Grids were rinsed with 20 drops of 0.1 M phosphate buffer. Antiserum-coated grids were placed on drop of purified virus for 5 min and rinsed with 20 drops of 0.1 M phosphate buffer. Grids were stained with a drop of 2% uranyl acetate for 30 sec. The decorated virus particles were observed on a Carl Zeiss LEO 906 transmission electron microscope.

Indirect enzyme linked immuno sorbent assay (ELISA). For selection of hybridoma cell lines, activity of MAbs obtained from fusion plate was measured by indirect ELISA. Wells of 96-well plates were coated with 100 μl of antigen solution containing 250 ng/ml purified recombinant protein in carbonate-bicarbonate buffer, pH 9.6 and then incubated at 37°C for 1 h. After removing antigens, wells were blocked with 2% skim milk, incubated at 37°C for 1 hr, and then washed 3 times with PBS containing 0.1% Tween 20 (PBST). Wells were filled with 100 il of antibody and then incubated at 37°C for 2 hr. After incubation with Horseradish peroxidase conujugated anti-mouse immunoglobulin diluted 1:5000 in PBS for 2 h at 37°C, plates were washed 5 times with PBST. Plates filled with orthophenylenediamine dihydrochloride substrate solution were incubated at room temperature for 10 min. The ELISA reactions were recorded using an ELISA reader at 492 nm.

Western blotting. Specificity of the MAbs and PAbs was evaluated by Western blot using healthy and LMoV-infected lily, and PVY-infected tobacco maintained in greenhouse. Plant tissue extract prepared by grinding 0.1 g of lily leaf in 1 ml PBS buffer, and separated by 12.5% SDS-PAGE were electroblotted to a nitrocellulose membrane (Sigma-Aldrich, USA). After blotting, the membranes were incubated for 1 h in 5% skim milk in PBST followed by adding 1:200 dilution of antibodies (2 mg/ml). The membranes were incubated overnight at 4°C. After washing two times with PBST, the membranes were incubated with anti-mouse antibodies conjugated with alkaline phosphatase (Promega, USA) in 5% skim milk in PBST for 2 h at room temperature. After washing two times with PBST, LMoV

specific bands were visualized with NBT/BCIP as a substrate (Sigma-Aldrich, USA).

Double antibody sandwich (DAS)-ELISA. Alkaline phosphatase conjugated antibody was prepared by using alkaline phosphatase labeling kit-SH with 2.0 mg/ml IgG (Dojindo, Japan), according to the manufacturer's instructions.

Sensitivity and specificity of the PAbs and MAbs were evaluated by DAS-ELISA. Wells of EIA/RIA plate (Costar, USA) were coated with antibodies (2 mg/ml) diluted in 1:200. After 4 h incubation at 37 °C, the wells were washed three times with PBST and loaded with the 200 μl sample of plant tissue extract prepared by grinding 0.1 g of lily leaf in 1 ml PBS. The plate were incubated overnight at 4 °C, washed extensively with PBST, and loaded with mouse anti-LMoVK-LiCB-CP antibody alkaline phosphatase conjugate diluted 1:200, 1:500, 1:1000, 1:2000, 1:5000 and 1:1000 (v/v). After 4 h incubation at 37 °C, the plate were washed extensively with PBST. The wells were filled with substrate solution, p-nitrophenyl phosphate (Sigma-Aldrich, USA), incubated at room temperature for 30 min, and the ELISA reactions were recorded using an ELISA reader at 405 nm.

Results

Amplification of CP of LMoVK-LiCB. CP gene of LMoVK-LiCB amplified by PCR showed about 825 bp PCR product (Fig. 1). The nucleotide sequences of CP gene were deposited under the GenBank accession no. of EU267778.

Production of recombinant protein in *E. coli*. The expressed CP was analyzed by SDS-PAGE in which the presence of a band with a molecular mass corresponding to approximately 74.4 kDa, an expected value for the fusion protein, was observed (Fig. 1). Capability 30.8 kDa corresponding to the LMoV-CP itself, increased by 43.6 kDa due to His-MBP tagging.

Production of MAbs and typing. Spleens of five immunized mice were used in three independent fusions. Of the 480 possible hybridoma cell lines assayed, 7 were identified that produced antibodies specific to LMoVK-LiCB-CP. Two of these were finally selected through subcloning twice by limiting dilution, and their isotypes were determined by mouse monoclonal antibody isotyping kit (Pierce, USA) (Table 1). Mouse MAb isotyping kit showed that IgG_{2b} and Kappa chain class antibodies gave strong reactivity in both hybridoma cell lines #2 and #7.

Testing the antibodies for the specific detection of LMoV. ISEM showed that MAbs decorated only LMoVK-LiCB particles. They did not react to PVY, serologically unrelated

(A)
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MHHHHHHMKI EEGKLVIWIN GDKGYNGLAE VGKKFEKDTG IKVTVEHPDK LEEKFPQVAA  60
TGDGPDIIFW AHDRFGGYAQ SGLLAEITPD KAFQDKLYPF TWDAVRYNGK LIAYPIAVEA 120
LSLIYNKDLL PNPPKTWEEI PALDKELKAK GKSALMFNLQ EPYFTWPLIA ADGGYAFKYE 240
NGKYDIKDVG VDNAGAKAGL TFLVDLIKNK HMNADTDYSI AEAAFNKGET AMTINGPWAW 300
SNIDTSKVNY GVTVLPTFKG QPSKPFVGVL SAGINAASPN KELAKEFLEN YLLTDEGLEA 360
VNKDKPLGAV ALKSYEEELA KDPRIAATME NAQKGEIMPN IPQMSAFWYA VRTAVINAAS 420
GRQTVDEALK DAQTNSSSNN NNNNNNNNLG IEGRGSANET LNAGASSSTQ TSRPTRPETA 480
AVDVAPQQSP ESRVRDRDVD AGTVGTFRIP RLKALATKIS IPKIRGRTIV NLGHLVNYNP 540
EQTDISNTRS TQKQFETWYN AVKDEYGLND ESMALTMNGL MVWCIENGTS PNINGAWLMM 600
DGDQQVEFPL RPIVEHAKPT LRQIMAHFSH LAEAYIEKQN AEKPYMPRYG LQRNLTDFTL 660
ARFAFDFYEV TSRTPARAKE AHFQMKTAAL RGKQSKLFGL DGKVTTQDED TERHTADDVN 720
KNMHSLLGIS M                                                     731
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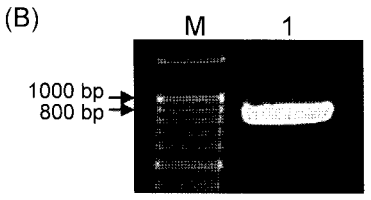

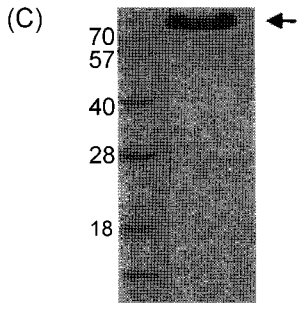

Fig. 1. Amino acid sequences of pRSET-LMOVK-CP. (A) Amino acid sequences of His-MBP region (under lined letters) and coat protein of LMoVK-LiCB. (B) Electrophoresis of PCR product amplified from LMoVK-LiCB using a primer pair LMOV-F and LMOV-R in 1% agarose gel. About 825 bp PCR product are shown (arrow) in lane 1. M: 1 kb DNA ladder. (C) Analysis of recombinant coat protein of LMoVK-LiCB expressed in *E. coli* strain pLysS in 15% SDS-PAGE gel. Three ì was loaded per lane. About 74.4 kDa band is shown (arrow).

Table 1. Reactivity of the class and subclasses of antibodies with LMoVK-LiCB-CP monoclonal antibodies produced from hybridoma cell lines in indirect ELISA [a]

Antibody	Absorbance at 492 nm/hybridoma cell lines	
	# 2	# 7
IgG_1	1.0320	0.4160
IgG_{2a}	0.7620	0.6290
IgG_{2b}	2.1740	2.1500
IgG_3	0.4800	0.4920
IgM	0.3410	0.3710
IgA	0.4650	0.4510
Kappa chain	2.2550	2.1950
Lambda chain	0.4930	0.4520

[a] The test for each cell line consisted of coating each well with 250 ng LMoVK-LiCB-CP recombinant protein, followed sequentially by 1:000 dilution of antibody, a 1:5000 dilution of horseradish peroxidase-conjugated goat antimouse antibody, and substrate. Isotyping was conducted with mouse monoclonal antibody isotyping kit (PIERCE, USA).

virus, confirming that MAbs are specific to LMoV (Fig. 2).

Testing the MAbs and PAbs for the specific detection of LMoV by Western blot revealed that it reacted strongly with extracts from LMoV-infected lily. A band with a molecular mass corresponding to approximately 30 kDa was observed in both membranes (Fig. 3A, Fig. 3B). The antisera did not react to healthy samples or non-serelogically related virus PVY. In DAS-ELISA, the MAbs reacted with leaf extracts from infected lily in dilutions of up to 1:10000 of conjugate (Table 2). The antibodies did not show heterologous cross reactions with other non-serologically related viruses (Table 2). Meanwhile PAbs failed to react in DAS-ELISA. Both LMoV-infected lily and healthy control gave A405 nm readings of 0.1-0.2 with PAbs (data not shown). Meanwhile, 0.1 mg/ml purified virus from LMoV-infected lily diluted 1:25 gave A405 nm reading of 1.1 to 0.4 (data not shown).

Discussion

The expression of viral CP in *E. coli* has been reported for a large number of plant viruses (Barbieri et al., 2004; Čeřovská et al., 2003; Chen et al., 2002; Fajardo et al., 2007; Jelkmann and Keimkonrad, 1997; Ling et al., 2000; Nikolaeva et al., 1995; Petrzik et al., 2001; Vaira et al., 1996). These antisera raised from animals have been

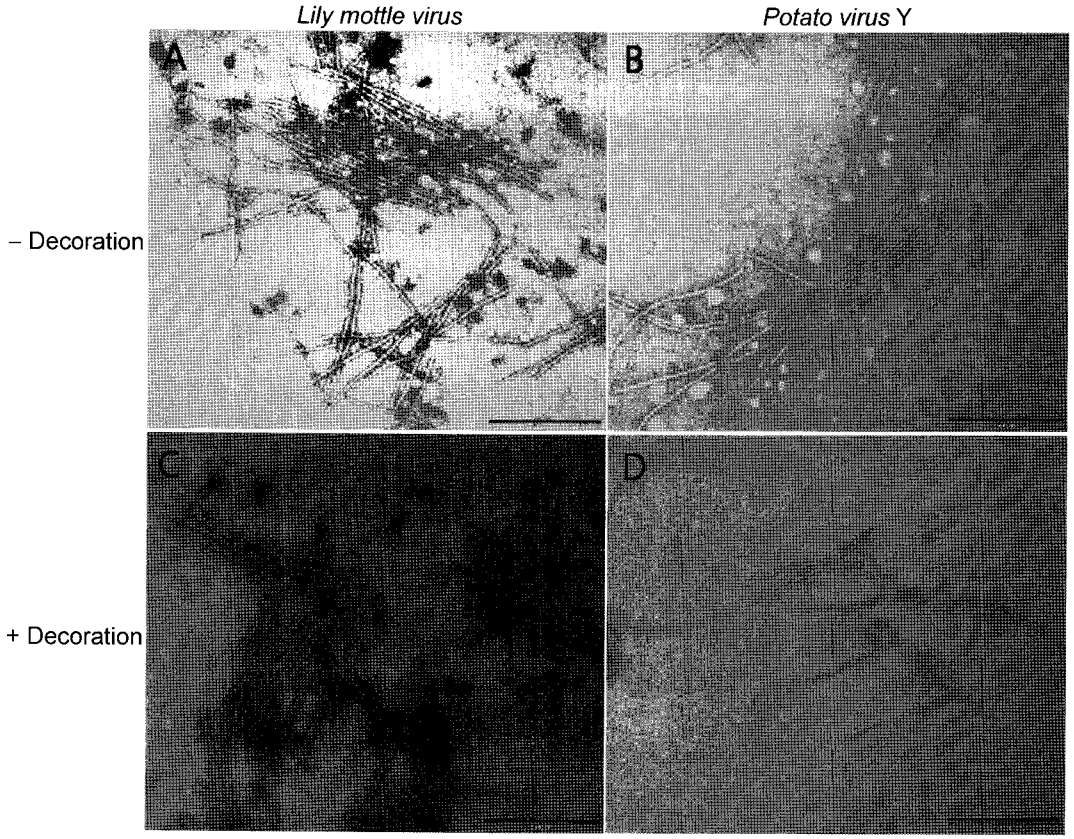

Fig. 2. Decoration of LMoV particles by MAbs observed under the electron microscope. (A) and (B) were a negative staining showing virus particles LMoV and PVY, respectively, non-decorated particles. MAbs decorated LMoV (C), but did not react with a *Potato virus* Y (D), non-serologically related virus.

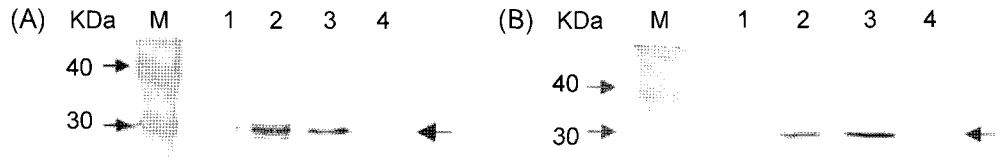

Fig. 3. Western blot analysis with antibodies against the LMoVK-LICB-CP fused to a 6 His-tag and MBP (1:250 v/v). (A) Western blot with PAbs. Lane 1, healthy lily; lane 2, LMoV-infected lily (1:10 w/v); lane 3, LMoV purified from infected lily (1:100 v/v, 0.1 mg/ml); lane 4, PVY-infected lily (1:10 v/v). (B) Western blot with MAbs. Lane 1, healthy lily; lane 2, LMoV-infected lily (1:10 w/v); lane 3, LMoV purified from infected lily (1:100 v/v, 0.1 mg/ml); lane 4, PVY-infected lily (1:10 v/v). The arrow on the right indicates the LMoV-CP.

Table 2. Results of DAS-ELISA with *Lily mottle virus* (LMoVK-LiCB)-infected lily extract using monoclonal antibodies raised against LMoVK-LiCB recombinant protein, expressed in *E. coli*

Samples (1:10 w/v)	Absorbance at 405 nm/conjugate (v/v)					
	1:200	1:500	1:1000	1:2000	1:5000	1:10000
Healthy lily	0.354	0.242	0.185	0.161	0.147	0.133
LMoV-infected lily	3.741	3.508	3.713	3.755	3.311	3.131
CMV- infected lily	0.354	0.276	0.185	0.155	0.138	0.125
LSV- infected lily	0.303	0.212	0.183	0.153	0.149	0.137
PYV-infected tobacco	0.367	0.249	0.188	0.156	0.146	0.135

successfully used for detection of plant viruses using ELISA and Western blot analysis, but some of them have cross-reacted to antigens in DAS-ELISA (Čeřovská et al., 2003; Jelkmann, 1997). There are few recent reports, in which antibodies produced against viral proteins of *Tomato spotted wilt virus* (TSWV), *Grapevine leafroll* associated *closterovirus*-3 (GLRaV-3) and *Prunus necrotic ringspot virus* (PNRSV) expressed from *E. coli* were found to be effective in detecting the corresponding viruses using DAS-ELISA (Fajardo et al., 2007; Ling et al., 2000; Petrzik et al., 2001; Vaira et al., 1996). It is clearly shown that PAbs and MAbs raised against recombinant CP of LMoVK-LiCB isolate were effective to detect LMoV isolates that existed in lily plants using Western blot analysis. In general, previous extensive studies showed that molecular weight of CPs of potyviruses ranged from 30 to 37 kDa (Shukla and Ward., 1989). In accordance with this criterion, molecular weight of single 30 kDa protein detected predominantly using antisera was CP of LMoV. It is coincident with expectation of molecular weight of LMoVK-CP of 30.877 kDa deduced from 275 amino acid sequence, suggesting that these antisera have specificity against LMoV isolates and can be used for specific detection of the virus.

We observed that MAbs were more sensitive than PAbs for detection of LMoV isolates present in lily plants using DAS-ELISA, since PAbs could not discriminate LMoV-infected lily from healthy control. Our result was consistent with the findings of Monis and Bestwick (1997) where the polyclonal antibodies could not differentiate viral infected tissues from healthy tissue using ELISA (Harlow and Lane, 1988). DAS-ELISA using PAbs results showed absorbance value from purified virion was greater than aborbance value from the diseased lily tissue (data not shown), suggesting that the sensitivity of PAbs raised against recombinant LMoVK-LiCB-CP in DAS-ELISA was not sufficient for detection of LMoV in lily tissues. We concluded that the recombinant viral CP expressed from bacterial cells has a great advantage for raising specific antibodies to plant viruses. Monoclonal antibodies against the coat protein of LMoVK-LiCB produced by such system were useful for detection of the virus on disease lily plants for future field researches.

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