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Incidence of *Lettuce mosaic virus* in lettuce and its detection by polyclonal antibodies produced against recombinant coat protein expressed in *Escherichia coli*

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A B S T R A C T

Lettuce mosaic virus (LMV), a member of the genus Potyvirus of family *Potyviridae*, causes mosaic disease in lettuce has recently been identified in India. The virus is seed borne and secondary infection occurs through aphids. To ensure virus freedom in seeds it is important to develop diagnostic tools, for serological methods the production of polyclonal antibodies is a prerequisite. The coat protein (CP) gene of LMV was amplified, cloned and expressed using pET-28a vector in *Escherichia coli* BL21DE3 competent cells. The LMV CP was expressed as a fusion protein containing a fragment of the *E. coli* His tag. The LMV CP/His protein reacted positively with a commercial antiserum against LMV in an immunoblot assay. Polyclonal antibodies purified from serum of rabbits immunized with the fusion protein gave positive results when LMV infected lettuce (*Lactuca sativa*) was tested at 1:1000 dilution in PTA-ELISA. These were used for specific detection of LMV in screening lettuce accessions. The efficacy of the raised polyclonal antiserum was high and it can be utilized in quarantine and clean seed production.

1. Introduction

LeBTuce (*Lactuca sativa*) is the most economically important vegetable crop belonging to family *Composite* (Krause-Sakate et al., 2001) and typically eaten cold, raw, in salads, sandwiches, hamburgers, tacos and in many other dishes throughout the world (Fletcher et al., 2005). Lettuce mosaic is the main viral disease of this crop and was identified more than 80 years ago (Jagger, 1921) and slowly became a worldwide serious economic problem largely due to its seed borne nature (Newhall, 1923; Ryder et al., 2003). In India also, natural occurrence of LMV was reported on lettuce recently (Sharma et al., 2013). The causal organism is *Lettuce mosaic virus* (LMV), a member of the genus potyvirus in the family *Potyviridae*, it is seed-borne and disseminated by aphids in a non-persistent manner by *Myzus persicae* and *Macrosiphum euphorbiae* (Retana et al., 2008). LMV consists of flexuous virus particles of 680–900 nm × 11–15 nm, encapsidating a monopartite positive ssRNA genome with a VPg at the 5′ end and a 3′ poly-A tract expressed as a polyprotein that cleaves to functional proteins (Lopez-Moya and García, 1999; Hull, 2002). Symptoms caused by LMV vary considerably depending on the genotype, infective strain or stage of infection and environmental conditions. The characteristic symptoms on susceptible lettuce cultivars include dwarfism, mosaic, leaf distortion, vein clearing and yellowing of the leaves with sometimes a much reduced heart of lettuce leading to failure to form heads and necrotic reactions (Dinant and Lot, 1992; Revers et al., 1997; Candresse et al., 2007). Among the various diagnostic techniques, the immuno-based detection has been routinely used for virus detection (Hull, 2002). Availability of good qualitative polyclonal antibodies is important to achieve specificity and sensitivity. The use of purified virus preparations is usually a time consuming procedure and presents problems with regard to purity and difficulty in obtaining a high concentration of virus particles (Carvalho et al., 2013). To overcome this problem, recombinant proteins expressed in prokaryotic systems such as *Escherichia coli* are frequently used in research, being stable, abundant and easily purified (Hull, 2002; Alkowni et al., 2011; Khatabi et al., 2012. In the present study, we describe the cloning and expression of recombinant fusion protein containing the complete coat protein (CP) of LMV with a His-tag followed by the production of polyclonal

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antibodies. The antibodies were subsequently used for detection of LMV using plate trapped antigen (PTA)-ELISA for screening lettuce accessions.

2. Materials and methods

2.1. Virus source, PCR amplification and cloning

A LMV clone containing the 3′ terminal region of nucleotide sequence consisting of the partial NIb, complete CP and 3′UTR (GenBank, NCBI accession no. JQ794776) originally isolated from lettuce, (Sharma et al., 2013) was used for designing primers for amplifying the full length CP. The primer pair LMVCPF: 5'***CCATGG***GAATGGTAGACGCAAAGCTT3' and LMVCPR: 5′ ***GAATTC***GTGCAACCCTCTCACGCC3′ with *Nco*I and *Eco*RI restriction sites respectively (bold italics) were designed for directional cloning. PCR profile consisted of a single cycle of 2 min at 95 °C, 30 cycles of 30 s at 94 °C, 45 s at 55 °C and 1 min at 72 °C; and a final extension for 10 min at 72 °C. Amplified products were analyzed by agarose gel electrophoresis (1.2% gel). PCR amplicons were purified from agarose gel using SV wizard PCR clean up system (Promega) and recloned into pGEMT-Easy vector (Promega), then transformed into *Esherichia coli* strain DH5α following standard protocols (Sambrook and Russell, 2001). Recombinant clones were identified using colony PCR, plasmid PCR and restriction digestion of plasmid with *NcoI* and *EcoRI*.

2.2. Production of polyclonal antibodies

2.2.1. Construction of prokaryotic expression vector pET-28a-LMV_CP

Coat protein cloned into pGEM-T easy vector was double digested with *NcoI* and *EcoRI* restriction enzyme and the resulting fragment was cloned into *NcoI* and *EcoRI* digested pET-28a expression vector generating pET-28a-LMV_CP. The recombinant DNA was transformed into an *E. coli* BL21 (DE3 strain) to express His-tagged LMV_CP.

2.2.2. Expression and purification of recombinant pET-28a-LMV_CP

The *E. coli* strain BL21 was transformed with the recombinant plasmid construct (pET-28a-LMV_CP) and selected on LB agar plate supplemented with 30 μg/ml of kanamycin. The transformants were grown in 5 ml LB containing 30 μg/ml kanamycin at 37 °C, 200 rpm for 2 h and induced by isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM for an additional 3 h. The expressed recombinant protein was analyzed by SDS-PAGE (Laemmli, 1970) using 5% stacking and 12% resolving gels. Electrophoresis was done at 95 mA for 2 h and gels were stained with coomassie brilliant blue R 250 stain (Sigma, St. Louis, USA). Protein was mixed with 80 μl of 2× SDS loading dye containing β-mercaptoethanol and denatured at 100 °C for 5 min prior to PAGE. An aliquot of 10 μl was analyzed in 10% SDS-PAGE. An uninduced control culture was analyzed in parallel. Similarly, expression was carried out at large scale with 2 litres of LB for purification. The crude expressed protein was loaded into the SDS gel along with the pre-stained marker and after electrophoresis the band containing the expressed protein was cut with sharp sterilized scalpel blade, crushed in 10% SDS and stored at 0 °C overnight followed by centrifugation. The clear supernatant acted as pure protein. It was dialysed using small wonder lyser kit (Promega, USA) to remove the impurities and quantified using a Nanodrop™ 1000 spectrophotometer (ThermoScientific, USA) and subsequently used for western blot and immunization.

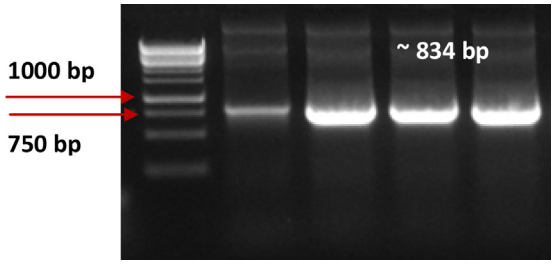

Fig. 1. An agarose gel following electrophoresis showing PCR amplification of CP gene from a LMV positive clone. M: 1 kb DNA ladder (Fermentas), Lanes1–4: LMV CP amplicon.

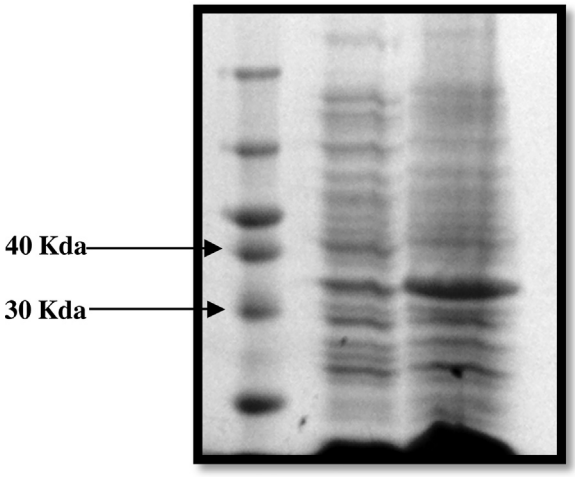

Fig. 2. An SDS PAGE gel following electrophoresis showing the expression of the LMV CP cloned in pET-28a. Lane 1: Protein prestained ladder, Lane 2: Uninduced pET-28a, Lane 3: Induced pET-28a.

2.2.3. Western blotting

The specificity of the expressed His-LMV_CP fusion protein was determined using commercial polyclonal antibodies (PAbs) to LMV (Agdia, 1:200) in western blot (O'Donell et al., 1982). The purified recombinant protein separated by SDS-PAGE gel was transferred to nitrocellulose membrane (Porablot NC membrane, Macherey-Nagel, Germany) using iblot (Invitrogen). The membrane was incubated with blocking solution (5% BSA in PBS) for 1 h at 37 °C. After 3 washings with PBS, the membrane was incubated with LMV polyclonal antibodies (PAbs). The membrane was washed three times and incubated with goat antirabbit IgG alkaline phosphatase-conjugated secondary antibody at 1:30000 dilution (Sigma, USA). Detection was accomplished by the addition of 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (BCIP/NBT) substrate solution (Genei, Bangalore, India).

2.2.4. Immunization of rabbit

Immunization of white New Zealand rabbit was performed by injecting the purified recombinant LMV_CP/His fusion protein (200 μg) emulsified with Freund's complete adjuvant (1:1 v/v; Santacruz, USA) intramuscularly into the hind legs of the rabbit followed by 4 injections (100 μg) emulsified with Freund's incomplete adjuvant (Genei, Bangalore India) at weeks interval after 15 days of 1st injection.

2.2.5. Antiserum collection

The rabbits were bled three times (one bleed per week) after one week of the 5th injection. Blood was collected from the ear vein, settled for 1 h at room temperature and keptat 4 °C overnight. Serum was separated after overnight incubation by centrifuga-