Phytopathology Research

RESEARCH Open Access

Development of polyclonal antiserum against movement protein from *Potato leafroll virus* and its application for the virus detection

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Abstract

The serological method is one of the most important techniques extensively used in crop production to detect different pathogens, especially plant viruses. An antiserum is essential for serological tests. The 17 kDa movement protein (MP) of *Potato leafroll virus* (PLRV) is related to the membranous structures and localized to the plasmodesmata, but there is no report on preparation of PLRV-MP antiserum for detection of PLRV. To prepare PLRV-MP antiserum, reverse transcription polymerase chain reaction (RT-PCR) was carried out to amplify the PLRV-MP gene, which was constructed into a prokaryotic vector to express the protein in *Escherichia coli* for immunization of rabbits, after purification. Western blotting revealed that this developed antiserum could effectively detect PLRV, but with better results from the perspective of color development and economics by using antiserum at the ratio range of 1:10000 to 1:40000, presenting high sensitivity and specificity to PLRV. The serological detection results for PLRV of the field samples were identical to the RT-PCR detection. This is the first report on the development of PLRV-MP antiserum that has been successfully used for both laboratory and field detection of PLRV. The results provide a fundamental tool for further research on the function of PLRV-MP.

Keywords: *Potato leafroll virus*, MP, Prokaryotic expression and purification, Antiserum development, Serological detection

Background

Potato (*Solanum tuberosum* L.) is one of the most important crops and is cultivated in more than 100 countries all over the world (He et al. 2012). It is infected by many pests, including at least 40 viruses and two viroids (Jeffries et al. 2006). One of the most severe viral diseases is caused by PLRV, which is distributed widely in the potato-growing areas of the world (Gillen and Novy 2007). PLRV is a representative species of the genus *Polerovirus* belonging to the family *Luteoviridae* (King et al. 2012) and was first described by Quanjer et al. in 1916 (Loebenstein et al. 2001). It is transmitted by aphids in a circulative non-propagative manner and is mainly restricted to the phloem tissues of infected plants (Mayo and Ziegler-Graff 1996; Lee et al. 2005). This viral disease is reportedly responsible for individual plant yield losses of over 50% and causes an annual global yield loss of 20 million tonnes (Wales et al. 2008). Primary symptoms of PLRV infection are yellowing of leaves, which may roll inward (Batool et al. 2011). Secondary symptoms in plants grown from infected potato tubers are stunting of shoot and leaflets rolling upwards, starting with the oldest leaves (Warren et al. 2005).

PLRV is a positive-sense single-stranded RNA virus comprising approximately 5.9 kb with six ORFs (open reading frame) encoding for proteins (King et al. 2012). The fourth ORF encodes a 17 kDa phloem-limited protein (P4, or MP) that is associated with virus cell-to-cell movement (Sokolova et al. 1997) and also has a role in regulation of PLRV replication (Tacke et al. 1991). The PLRV-MP can form homodimers and this dimerization

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occurs in the N-terminus (Tacke et al. 1993). The PLRV-MP is phosphorylated near its C-terminus by a membrane-associated protein kinase, occurring in membranous structures possibly at the deltoid plasmodesmata (Sokolova et al. 1997).

Serology testing is one of the most important techniques widely used in plant virology for its specificity in disease diagnosis and relative ease of accomplishment (Derrick 1973; Lin et al. 1990). The effectiveness of a successful serological detection mostly depends on the availability and specificity of the antiserum. Most plant viruses contain MP genes in their genome that are associated with cell-to-cell movement of the virus through the plasmodesmata (Wolf et al. 1989; Haupt et al. 2005; Akamatsu et al. 2007). Anti-MP antisera used to detect these MPs in different plant viruses have been reported (Xie et al. 2007; Calegario et al. 2012; Li et al. 2015; Koolivand et al. 2016). As far as we know, there is no report on the preparation of PLRV-MP antiserum used for the detection of PLRV and its MP. Therefore, our study was conducted to purify bacterially-expressed recombinant PLRV-MP fusion protein and prepare its specific antiserum, which was successfully used to detect the MP in PLRV-infected plants.

Results

Prokaryotic expression and purification of PLRV-MP recombinant protein, and preparation of polyclonal antiserum

The PLRV-MP gene was amplified by PCR, and ligated with a prokaryotic expression vector pDB.His.MBP to produce pDB.His.MBP–PLRV-MP. Then the MP fusion protein was expressed in *Escherichia coli* (strain: BL21) and purified. During the prokaryotic expression and purification of PLRV-MP protein, the osmotic fluid was detected using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The results showed a specific protein band near the 67 kDa protein marker (Fig. 1a), consistent with the size of the His.MBP–PLRV-MP fusion protein (Fig. 1b), indicating that the fusion protein was successfully purified and the His.MBP tag was banded at 42 kDa (Fig. 1b). Perhaps due to the rupture of the PLRV-MP and His.MBP tags, the fusion protein degraded during the purification process. The concentration of the fusion protein was approximately 2 mg/mL. The purified fusion protein (4 mL) was used to prepare the polyclonal antiserum in the Beijing Genomics Institute (Beijing, China), and a total of more than 50 mL of antiserum was obtained.

Titer analysis

The titer of PLRV-MP antiserum was analyzed by Western blotting with PLRV-infected *Nicotiana benthamiana* leaves. A weak protein band of 17 kDa (Sokolova et al. 1997; Lee et al. 2002) was detectable down to an antiserum dilution of 1:1280000. From the viewpoint of color development, the antiserum functioned best at the ratio range of 1:10000 to 1:40000 (Fig. 2).

Sensitivity analysis

In order to analyze the sensitivity of the PLRV-MP antiserum (anti-MP^{PLRV}), proteins extracted from the PLRV-infected *N. benthamiana* leaves were serially diluted and subjected to Western blot analysis. When the total protein was diluted by 40-fold, a positive band was clearly detected for the antiserum dilution factors 1:10000 and 1:20000 (Fig. 3a, b), while purified MP fusion protein of approximately 0.025 ng could be detected with the antiserum diluted to 1:20000 (Fig. 3c).

Specificity analysis

Total protein was extracted from *N. benthamiana* leaves infected by PLRV, *Turnip yellows virus* (TuYV) and *Brassica yellow virus* (BrYV), respectively, and healthy leaf

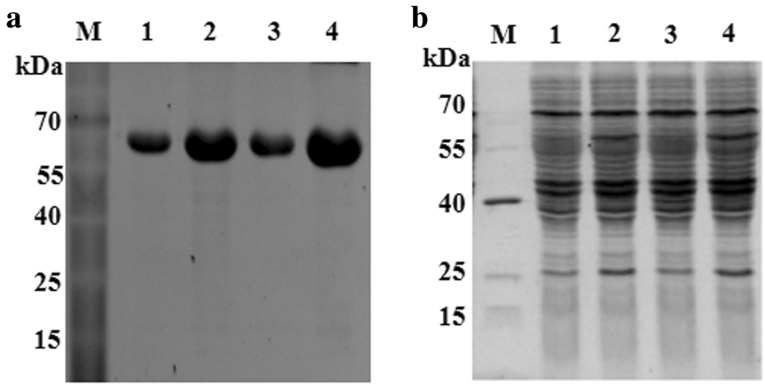

Fig. 1 SDS-PAGE analysis of PLRV-MP recombinant protein. **a** Lane M: PageRuler Prestained Protein Ladder, and lanes 1–4: Purified PLRV-MP recombinant protein. **b** Induced expression of PLRV-MP recombinant protein. Lanes 1, 3: Non-induced bacterial lysate, and lanes 2, 4: IPTG-induced lysate

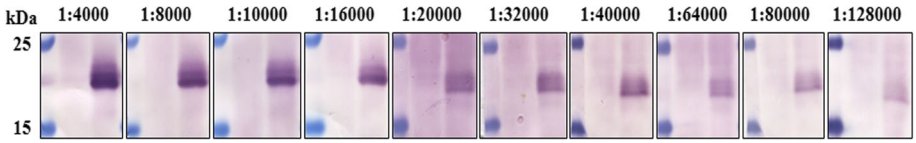

Fig. 2 Titer determination of PLRV-MP antiserum by Western blotting. Antiserum used at 10 different dilutions (1:4000, 1:8000, 1:10000, 1:16000, 1:20000, 1:32000, 1:40000, 1:64000, 1:80000, and 1:128000) against PLRV, each of which consists of a left lane of negative control and a right lane containing proteins extracted from PLRV-infected *Nicotiana benthamiana* leaves

was used as a negative control. Western blotting with anti-MP^{PLRV} at ratios of 1:10000, 1:20000, and 1:30000 revealed positive results only in reactions with PLRV-infected *N. benthamiana* leaf (Fig. 4), indicating that the antiserum was specific to PLRV.

Detection of PLRV from field samples using PLRV-MP antiserum and comparison with RT-PCR detection

Potato leaf samples from individual plants collected in Qianqin, Inner Mongolia were tested by Western blotting with anti-MP^{PLRV} at a ratio of 1: 20000. The positive bands exhibited in three leaf samples were consistent with that of the positive control (PLRV-infected *N. benthamiana* leaf) (Fig. 5a) and identical to that of RT-PCR detection (Fig. 5b), indicating that this anti-MP^{PLRV} was applicable for detection of PLRV in field crops.

Discussion

Protein expression is the way in which proteins are synthesized, modified and regulated in living organisms such as bacteria, yeast, plant or animal cells. The *E. coli* prokaryotic expression becomes a representative expression system as it has a clear genetic background, rapid reproduction, high expression levels, ease of purification of expression products, good stability, strong anti-pollution ability, low cost, and a wide range of applications (Hockney 1994). Many recombinant polyclonal antibodies specific to plant viruses have been previously generated for serological detection of viruses such as *Tomato spotted wilt virus* (Vaira et al. 1996), *Potato virus Y* (Folwarczna et al. 2008), *Alfalfa mosaic virus* (Khatabi et al. 2012), and *Wheat streak mosaic virus* (Tatineni et al. 2014). Recombinant antisera for Egyptian isolates of both *Potato virus X* (Soliman et al. 2006) and PLRV (Aseel and Hafez 2017; El-Attar et al. 2010) have also been produced. In our experiment, *E. coli* was used to express His.MBP–PLRV-MP from which PLRV-MP fusion protein was purified and anti-MP^{PLRV} successfully produced.

Viral MP plays an important role in the cell-to-cell movement of the infectious material. Recent research showed that the interaction of PLRV-MP with the protein 3a affected their localization to the mitochondria and plastids (DeBlasio et al. 2018). Serology is one of the

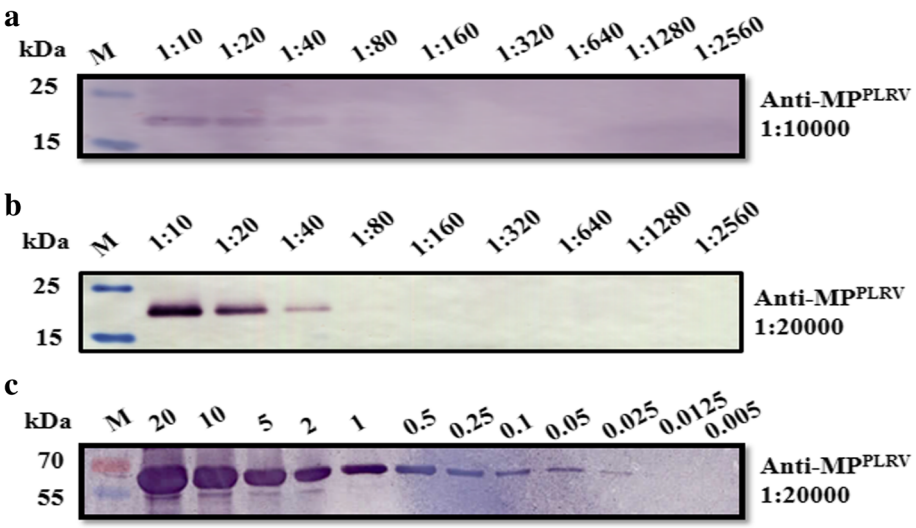

Fig. 3 Sensitivity analysis of PLRV-MP antiserum by Western blotting. **a**, **b** Lane M: PageRuler Prestained Protein Ladder, and the rest of the lanes show multiple dilutions of stock protein from the PLRV-infected *Nicotiana benthamiana* leaves (10-, 20-, 40-, 80-, 160-, 320-, 640-, 1280-, and 2560-fold), with anti-MP^{PLRV} at the ratio of 1:10000 and 1:20000 respectively. **c** Lane M: PageRuler Prestained Protein Ladder, and the rest of the lanes show different concentrations of purified MP fusion protein (20, 10, 5, 2, 1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.0125, and 0.005 ng), with antiserum at the ratio of 1:20000

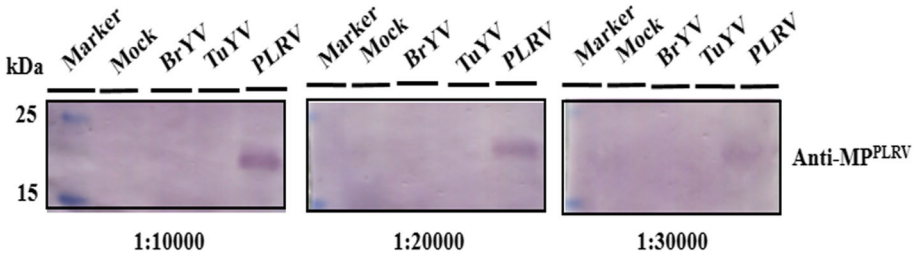

Fig. 4 Specificity analysis of PLRV-MP antiserum (anti-MP^{PLRV}) by Western blotting. Marker: PageRuler Prestained Protein Ladder, Mock: protein extracted from the healthy leaf was used as a negative control, and the rest of the lanes are for the protein extracted from *Nicotiana benthamiana* leaves infected by *Brassica yellow virus*, *Turnip yellows virus* and *Potato leafroll virus*, respectively

most important techniques for virus detection (Aseel and Hafez 2017) and the antiserum (either monoclonal or polyclonal) is the basis for the serology test. In most cases, the anti-coat protein (CP) antisera are used for virus detection; however, virus detection by the anti-CP antisera may not show whether the virus is in active (replication) or inactive stage. The positive detection of the MP with anti-MP antisera may indicate that the virus is in the multiplication stage. Polyclonal and monoclonal MP antibodies specific to *Barley yellow dwarf virus* (Xie et al. 2007; Li et al. 2015), polyclonal MP antibody specific to *Citrus leprosis virus* C (Calegario et al. 2012), and polyclonal MP antibody specific to *Grapevine fanleaf virus* (Koolivand et al. 2016) have been reported to detect the corresponding viruses, but most were effective only in laboratory use. Western blotting with anti-MP^{PLRV} in our study showed that the antiserum could detect PLRV efficiently, although ratios of 1:10000 to 1:40000 were better for color development. It was highly sensitive down to below 0.025 ng of protein at the antiserum ratio of 1:30000 and strictly specific to PLRV. The antiserum could also detect PLRV from field samples and the detection result was identical to that of RT-PCR.

Conclusions

Because viral MP plays an important role in the process of virus infection, efforts to express PLRV-MP in *E. coli* and to produce a specific polyclonal antiserum against this protein will provide an important tool for identification of PLRV infection and further research on the localization, expression, interaction with host and various biochemical modifications of PLRV-MP are needed.

Methods

Plant material and growth conditions

Wild-type *N. benthamiana* plants were grown at 24 ± 1 °C with a photoperiod of 16/8 h of light/dark cycle. Potato leaf samples were collected from Qianqi, Inner Mongolia.

Construction of PLRV-MP prokaryotic expression vector

The pT-PLRV-MP was constructed by amplification of pCa-PLRV containing the infectious cDNA clone of PLRV derived from the plasmid pBNUP110 (Franco-Lara et al. 1999) with the primer pair PLMPNdeF (5′-CATATGTCA ATGGTGGTGTACAA-3′) and PLMPXhoR (5′-CTCG AGTCATCCGCGCTTGATAAG-3′). The plasmids were then digested with *Nde*I and *Xho*I followed by ligation into

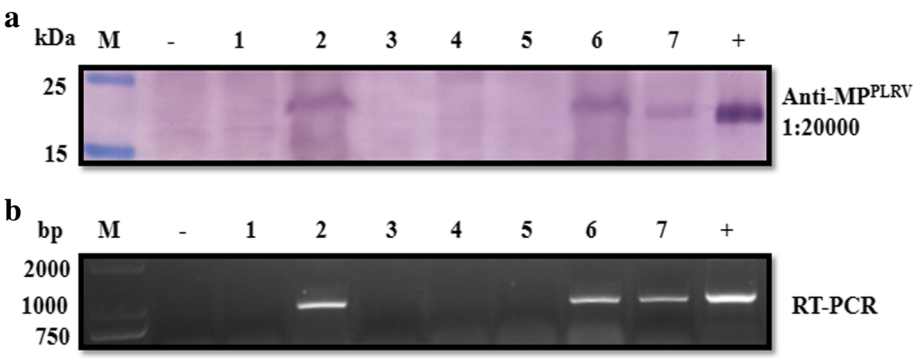

Fig. 5 Comparison of detections of PLRV from field crops using anti-MP^{PLRV} and RT-PCR. **a** Lane M: PageRuler Prestained Protein Ladder. **b** Lane M: DL2000 Ladder, lane "-": extract from healthy potato leaf (negative control), lanes 1–7: extracts from potato leaf samples collected from individual potato plants, lane "+": extract from PLRV-infected *Nicotiana benthamiana* leaf (positive control)

the expression vector pDB.His.MBP (DNASU Plasmid Repository, Arizona, USA), and predigested with *Nde*I and *Xho*I to obtain the prokaryotic expression clone pDB.His.MBP–PLRV-MP containing the target gene.

Prokaryotic expression and purification of PLRV-MP protein, and preparation of polyclonal antiserum

Oscillation culture of pDB.His.MBP–PLRV-MP was adjusted to an OD_{600} of 0.6–0.8. Isopropyl-β-D-thiogalactoside (IPTG; Sigma-Aldrich, St. Louis, MO, USA) was added to make a final concentration of 0.1 mM. After incubation at 18 °C for 6 h, it was centrifuged at 4000×g for 10 min. The cells were resuspended with high-salt buffer (20 mM Tris-HCl, 500 mM NaCl, pH 8.0) and centrifuged at 12,000×g for 1 h. The supernatants were collected on a Ni-affinity column (Qiagen, Hilden, Germany) and the proteins washed with elution buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8. 0). Each purification process was subjected to SDS-PAGE and the appropriate eluent was concentrated to obtain the pDB.His.MBP–PLRV-MP fusion protein.

The purified fusion protein was sent to the Beijing Genomics Institute to prepare the polyclonal antiserum (anti-MP^{PLRV}) by immunizing rabbits. Sensitivity and specificity of the antiserum developed against recombinant PLRV-MP were evaluated by Western blotting.

Western blot detection

Proteins were separated by SDS-PAGE using the protocol of Zhuo et al. 2014. Briefly, proteins were transferred to a nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) by electrotransfer (200 mA, 90 min) with a mini trans-blot electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA), and the nitrocellulose membranes were incubated in 1 × TBST buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) containing 5% skim milk powder at 37 °C. After blocking for 1 h, anti-MP^{PLRV} was added at a certain dilution and incubated at 37 °C for 1 h, and washed three times with 1 × TBST for 10 min each time. The nitrocellulose membrane was then incubated with anti-rabbit in goat (IgG; Sigma-Aldrich) diluted at 1:3000 for 1 h at 37 °C as a secondary antibody followed by washing with 1 × TBST. The band was visualized by using nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyphosphate (Sigma-Aldrich).

Determination of anti-MP^{PLRV} titer

Total protein was extracted from PLRV-infected *N. benthamiana* leaves and healthy *N. benthamiana* leaf (as a negative control) as described by Zhuo et al. 2014. Anti-MP^{PLRV} with a dilution factor of 4000, 8000, 10000, 16000, 20000, 32000, 40000, 64000, 80000, and 128000 was used in Western blotting to determine the titer value.

Sensitivity analysis of the anti-MP^{PLRV}

The experiment was performed by extracting the total protein from PLRV-infected *N. benthamiana* leaves and multiple dilution samples were prepared by diluting the stock sample multiples of 10, 20, 40, 80, 160, 320, 640, 1280, and 2560 with protein extraction buffer. Western blotting was performed using anti-MP^{PLRV} at the ratio of 1:10000 and 1:20000 respectively.

Another experiment was performed by loading 20, 10, 5, 2, 1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.0125, and 0.005 ng of purified protein respectively and anti-MP^{PLRV} was diluted at the ratio of 1:20000.

Anti-MP^{PLRV} specificity analysis

Total protein was extracted from *N. benthamiana* leaves infected by BrYV, TuYV and PLRV respectively with healthy leaf as a negative control. Specificity of the antiserum was detected by Western blotting.

Field application of the antiserum

Leaf samples collected from individual standing field potato plants in Qianqi, Inner Mongolia, were tested for PLRV detection using our newly developed anti-MP^{PLRV}, with healthy leaf sample as a negative control and PLRV-infected *N. benthamiana* leaf as a positive control. The result was compared with RT-PCR detection results.

Abbreviations

BrYV: *Brassica yellow virus*; CP: Coat protein; IPTG: Isopropyl-β-D-thiogalactoside; MP: Movement protein; ORF: Open reading frame; PLRV: *Potato leafroll virus*; RT-PCR: Reverse transcription polymerase chain reaction; SDS-PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis; TBST: Tris-buffered saline; TuYV: *Turnip yellow virus*

Acknowledgments

We thank Professor Peter Palukaitis (Seoul Women's University) for providing the plasmid pBNUP110 containing the infectious cDNA clone of PLRV, Dr. David Baulcombe (John Innes Centre, UK) for providing *N. benthamiana* plants and Dr. Salah Bouzoubaa (University of Strasbourg, France) for providing *E. coli* strain MC1022.

Funding

This work was supported by the National Natural Science Foundation of China (31671995 and 31371909) and the 111 project (B13006).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

C-GH conceived the study and revised the manuscript. FY and MR performed the experiments. FY drafted the manuscript and MR wrote the manuscript. X-YZ constructed a plasmid. ZYZ, YW, DWL and JLY contributed reagents/materials/analysis tools. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests
The authors declare that they have no competing interests.

Received: 17 September 2018 Accepted: 20 January 2019
Published online: 08 February 2019

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