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Molecular cloning, prokaryotic expression and preparation of antiserum against Lily symptomless virus TGB1 protein

Running title: *Lily symptomless virus* TGB1 protein

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ABSTRACT

The triple gene block gene TGB1 was amplified by RT-PCR from lily leaves infected with Lily symptomless virus and cloned into prokaryotic expression vector pET-28a(+). The recombinant vector was transformed into Escherichia coli strain BL21 (DE3). On induction with isopropyl β-D-1-thiogalactopyranoside,TGB1 protein was highly expressed and the molecular weight was 29 kDa (including a His-tag-containing fusion). After protein purification by Ni2+-NTA affinity chromatography, a polyclonal antibody against TGB1 was raised in mouse. Western blot analysis showed that the antiserum reacted specially with the TGB1 protein of LSV. ELISA and RT-PCR confirmed that the antiserum reacted specially with lily leaves infected with LSV, and can be used for a rapid test for LSV. The antibody produced in this work may be used for future immunohistochemistry and functional study of TGB1.

KEYWORDS antiserum preparation; cloning; expression; *Lily symptomless virus*; *TGB1* gene

INTRODUCTION

Lily symptomless virus (LSV; genus *Carlavirus*) is an essential component of necrotic fleck disease of liliaceous plants. LSV is usually transmitted in a non-persistent manner by aphids, causing dwarf plants, deformity of leaves and flowers, and even non-flowering (Jia H, 2014; Zhang Y,2015). In lilies undergoing vegetative propagation, the detection rate of LSV infection is much more than 90% (Asjes C J,2000). Scientific detection and effective control of LSV is imperative. Lately, the use of antiserum combined with real-time fluorescent PCR has been widely adopted as a means of detection of plant viruses (Tavasoli M,2009). To prepare antiserum, immunization of mice with viral sap is generally performed. Meanwhile, expression of virus capsid protein genes in *Escherichia coli* has also been reported (Abdel-Sala A M, 2014).

The LSV genome encodes four proteins, including the RNA-dependent RNA polymerase (RdRp), the triple gene block (TGB1), the coat protein (CP) and an unknown protein (Singh A K , 2008). TGB1 is a multifunctional protein. One potentially important function is to mediate virus movement. *TGB1* has a full length of 1160 bp, encoding three polypeptide chains of 228,106 and 64 amino acid residues, respectively. The amino acid sequences contain an ATP binding region and a nucleic acid binding region, with RNA helicase and nucleic acid hydrolase activities (Pin-San XU,2012). Here, the LSV-*TGB1* gene was cloned and expressed in *E. coli*. The aim was to produce antiserum that is viable for testing for LSV, and, in future, to investigate the subcellular location(s) of LSV-TGB1 and to analyze the protein functions.

MATERIALS AND METHODS

Viruses and reagents

Lily plants used to amplify the LSV-*TGB1* gene and the His tag-encoding expression vector PET-28a(+) were maintained in our lab. *E. coli* DH5α, a modified Bradford protein assay kit, nickel affinity columns and alkaline phosphatase labeled sheep anti-rat IgG were purchased from Sangon Biotech (Shanghai) Co., Ltd., who also performed primer synthesis and DNA sequencing. Vector pMDTM18-T and restriction enzymes were from TaKaRa Bio Inc. Freund's adjuvant was from Sigma. *E. coli* BL21 (DE3) cells were purchased from Tiangen Biotech (Beijing) Co., Ltd.

Gene cloning and vector construction

For construction of the expression vector for LSV-*TGB1*, we used as a template the total RNA of lily infected with LSV. LSV-*TGB1* was amplified by PCR using synthetic oligonucleotide primers 5'-ATGGATGTTTTACTAAGTTTGTTGA-3' and 5'-CTAAAGGTGAACTTGTAGCACTTT-3'.The whole genome sequence of LSV-DL has been registered with the NCBI (GenBank accession number HM222522.1). PCR amplification products were ligated into pMDTM18-T and transformed into E. coli *DH5α* competent cells. Positive clones were picked and delivered to Beijing Genomics Institute for sequencing. The PCR product was digested with *Bam*HI and *Eco*RI and ligated into the same restriction sites of pET-28a(+). Then, the plasmid was transformed into the *E. coli* BL21 (DE3) expression strain. All constructs were confirmed by restriction enzyme digestion and DNA sequencing.

Protein expression and purification

A single colony was inoculated into LB liquid medium. Expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C for 6 h. The fusion protein LSV-TGB1 with a polyhistidine tag was visualized by 12% sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE).

The precipitate was collected after cell disruption, and dissolved overnight in HCl. After centrifugation at 13400g for 20 min, the supernatant was removed and filtered (0.45-μm). Ni^{2+}-affinity chromatography was performed using the His-tag of the protein according to the instruction manual of the resin manufacturer (Sangon Biotech). The concentration of the protein solution collected was measured by the modified Bradford method. SDS-PAGE analysis was used to determine the purity and concentration concentration of the purified protein.

Mice and raising of a polyclonal antibody

Kunming mice (SPF) were purchased from the animal experiment center of Dalian Medical University. Eight female mice (18–20 g) were immunized four times. Six of the mice were given purified TGB1 protein, the other two PBS as a control. First, the purified protein was emulsified 1:1 in Freund's complete adjuvant, then injected intraperitoneally (0.5 ml/mouse). The first booster immunization was performed 2 weeks later, using Freund's incomplete adjuvant; this was repeated twice, the interval between procedures being 3 weeks. Three days after the final immunization, blood was collected from the eye venous plexus. The titer of the antiserum was determined by indirect enzyme-linked immunosorbent assay (ELISA).

Western blotting and ELISA

In western blotting, sheep anti-rabbit IgG conjugated to horseradish peroxidase, the recombinant TGB1 protein, and a 1:1 dilution of the antiserum as the primary antibody, were used. Equivalent amounts of protein were electrophoresed on 10% SDS-PAGE gels and transferred onto Immobilon P membranes. The membranes were blocked by incubation with 3% nonfat dry milk for 90 min at 37 °C and then incubated with primary antibodies overnight at 4 °C, then with the secondary antibody (1:2000).

ELISA analysis of six strains of LSV was carried out by using the antiserum as the primary antibody, and detected using peroxidase-labeled sheep anti-mouse antibody with AEC as the substrate. Meanwhile, the accuracy of the TGB1 antibody preparation in this ELISA was verified by RT-PCR assay.

RESULTS

Amplification of LSV-*TGB1* gene fragment

As Figure 1 shows, the RT-PCR product was ~700-bp, which is consistent with the expected size of the TGB1 gene".The fragment was cloned into vector pMDTM18-T, and the sequence was found to be identical to the NCBI database reference sequence, confirming that the fragment contained the *TGB1* gene.

Prokaryotic expression vector

Recombinant plasmid pET28-*TGB1* was verified by *Bam*HI and *Eco*RI double enzyme digestion; the resulting fragment was consistent with the expected size of 423-bp and thus the prokaryotic expression vector was constructed successfully (Figure 2). Sequencing results also proved that the fragment sequence was correct and could be used for the

following expression experiments.

Expression and purification of fusion protein

The expression plasmid was transformed into *E. coli* BL21 (DE3). Expression of recombinant protein was induced by IPTG and confirmed by SDS-PAGE; the recombinant protein was clearly visible on the gel at around the expected size of 29 kDa (including 4 kDa from the His-tag-fragment from pET-28a(+)) (Figure 3). The protein (Figure 4) was purified by nickel affinity chromatography and analyzed by SDS-PAGE. The purity and concentration of the protein (the latter determined by the Bradford method) were suitable for preparing antiserum.

Preparation and identification of TGB1 antiserum

KCl precipitation was used to collect the purified fusion protein, which was ground into powder and used as the antigen. Antiserum was obtained from immunized Kunming mice; the titer was up to 1:10000 by ELISA. Western blot analysis was carried out, which indicated that the antibody specifically recognized the TGB1 target protein (Figure 5).

ELISA and RT-PCR

ELISA was used to analyze six LSV lily disease samples. A color reaction was clear from all the LSV-infected samples, and there was no obvious color reaction from the leaves of healthy lily or the blank control (Figure 6). These results indicate that the prepared antiserum can identify the TGB1 protein, which can be used for the detection of LSV. Equivalent results were obtained by RT-PCR assay (Figure 7); thus the antiserum was reliable.

DISCUSSION

The use of *E. coli* to express foreign proteins has become one of the most economical and effective experimental techniques, and is used frequently (Wang R Y , 2010). LSV is a major bottleneck in the development of the lily planting industry. There is an urgent need to establish a fast and reliable detection technology for the virus. Serological detection is sensitive, rapid and suitable for large numbers of samples, and has been widely used to test for plant viruses (Bosque-Pérez N A,2014; Diaconescu M,2013). Usually, viral shell proteins are taken to prepare the antiserum (WU Qi-Yao,2013). However, in this study, we have successfully prepared TGB1-based antiserum against LSV by cloning the *TGB1* gene, expressing in a prokaryote and purifying the resulting fusion protein.

The sensitivity of the LSV-TGB1 antiserum was slightly lower than that of LSV-CP antiserum. This may be because the CP is the main part of the assembly of the virus particles, and its accumulation in the plant is higher than that of the TGB1 proteins. However, the virus was found in plant tissues of six infected strains by using the LSV-TGB1 antiserum in ELISA, and by RT-PCR, which indicated that the TGB1 antiserum was highly specific and accurate. Thus, the use of *E. coli* to express a foreign protein to raise an antibody is a simple and economical method. The results of this study may not only be applicable to the detection of LSV, but also make it possible to explore the functions of the TGB1 proteins, their cytological localization(s) and their interactions.

Until now, study of LSV has mainly focused on the pathogen, virus detection and protein bioinformatics (Xu et al.,2011). Lately, the need to investigate the functions of LSV proteins in the plant was highlighted, but this remains to be done. Because of the location, structure and size of the *TGB1* gene in the LSV genome, we suggest that this gene and the proteins it encodes may play a key role in the processes of virus infection, replication and translocation.

CONCLUSION

In this work, we successfully developed a simple and rapid method for preparing LSV-TGB1 antiserum for the first time. The antiserum was highly specific and sensitive for the rapid detection of LSV, and has great potential to be used for analysis of protein function, cytological localization and protein interactions.

Compliance with Ethical Standards:

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Conflict of Interest: All of the authors declare that they have no conflict of interest.

Ethical approval: All applicable international, national, and institutional guidelines for the care and use of animals were

followed.

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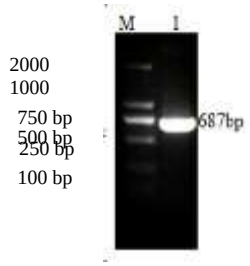

Fig.1. RT-PCR products of cloning of the *Lily symptomless virus* (LSV) *TGB1* gene.

Lanes: M. DL2000 DNA marker; 1. RT-PCR product containing the LSV *TGB1* gene fragment.

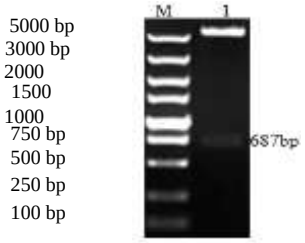

Fig.2. pET28-*TGB1* expression plasmid analyzed by enzyme digestion.

Lanes: M. DL2000 DNA marker; 1. *pET-TGB1* digested with *Bam*HI and *Eco*RI.

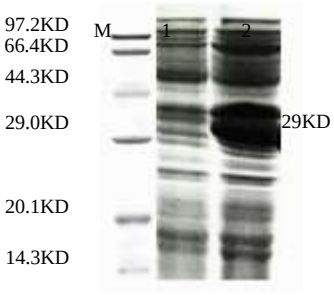

Fig. 3. SDS-PAGE analysis of LSV *TGB1* gene expression in *Escherichia coli* BL21 (DE3). Lanes: M. Protein molecular weight markers; 1. Proteins from non-induced *E. coli* transformed with pET28-*TGB1*; 2. Proteins from IPTG-induced *E. coli* transformed with pET28-*TGB1*.

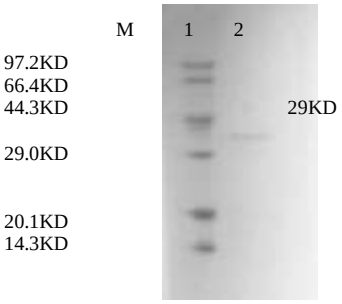

Fig. 4. SDS-PAGE analysis of purified LSV TGB1 His-tagged fusion protein. Lanes: M. Protein molecular weight markers; 1. Lysis supernatant of IPTG induced *E. coli* transformed with

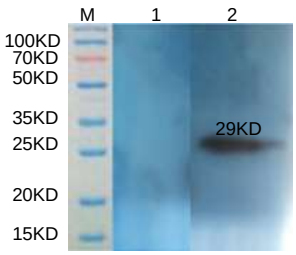

Fig. 5. Western-blot analysis of LSV TGB1 His-tagged fusion protein using mouse-raised antiserum.

Lanes: M. Protein molecular weight markers; 1. Negative (PBS-control) serum and recombinant TGB1 protein; 2. TGB1 antiserum and recombinant TGB1 protein.

Fig. 6. ELISA detection of LSV in lily.

1–6. LSV-infected lily samples; 7. Purified LSV TGB1 fusion protein; 8. Healthy leaves of lily; 9. Coating buffer control.

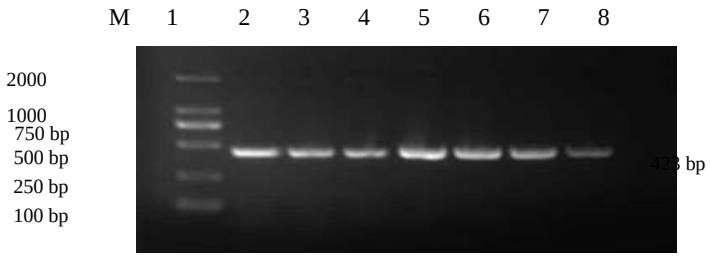

Fig. 7. RT-PCR detection of LSV in lily.

Lanes: M. DL2000 DNA marker; 1–6. LSV-infected lily samples; 7. Healthy leaves of lily; 8. The LSV *TGB1* gene fragment.