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MOLECULAR CLONING AND PROKARYOTIC EXPRESSION OF THE POLYPROTEIN GENE OF TULIP BREAKING VIRUS

NAN TANG[1, 2], RONGCHUN YE[1] AND DAOCHENG TANG[1, 2]*

[1]*Plateau Flower Research Centre, Qinghai University, Xining 810016, China*
[2]*Key Laboratory of Landscape Plants and Horticulture of Qinghai Province, Xining 810016, China*
**Corresponding author's email: tangdaocheng6333@163.com*

Abstract

The tulips are important bulbous ornamental plants. Tulip breaking virus (TBV) is the most serious virus disease of tulips which is transmitted by aphids. It is very important to pick out the infected bulbs before the aphids start spreading the virus. Notably, a fast and reliable diagnostic approach that can be carried out during the bulb storage period is needed to control the spread of TBV. Since the ELISA kit for the detection of TBV has not been commercialized, the aim of this study was to clone and express TBV protein in *E. coli* and prepare monoclonal antibodies for the virus. In the current study, the TBV polyprotein gene fragment *FK912_gp1* was obtained from TBV infected bulbs of the tulip cultivar 'Barcelona' by RT–PCR. *FK912_gp1* was successfully ligated with the expression vector pCold I and transformed into *E. coli*. The prokaryotic expression vector of pCold I-TBV was constructed. The fusion protein was expressed in *E. coli* and then purified, at a concentration of 1.24 mg/ml and a purity of greater than 90%. After cell fusion and subcloning, 2 hybridoma cell lines 2B12 and 4G6 were successfully obtained. The results of DAS-ELISA presented that the valence of the obtained monoclonal antibodies reached 512000. These results provide important theoretical support for future studies on the molecular structure, function, and immunogenicity of the TBV polyprotein.

Key words: TBV, Cloning, Prokaryotic expression, Virus detection.

Introduction

As an important ornamental plant worldwide, the tulips are favored by consumers due to their elegant and colorful flowers. Tulips have a long juvenile stage that takes approximately six years from seed germination to flowering. Therefore, tulips are mainly vegetatively propagated by dividing bulbs. A drawback of vegetative propagation is the spread of viruses. Tulip breaking is mainly caused by tulip breaking virus (TBV) which induces disordered synthesis and irregular distribution of anthocyanins in the petals and leaves of its host. The infected plants present variegated color patterns in the flowers and leaves. The virus not only decreases the ornamental value of the flowers but also reduces the reproductive capacity of the bulbs and thus causes yield reduction (De Kock *et al*., 2011).

TBV is a species of ssRNA virus belonging to the genus *Potyvirus* (Brandes & Wetter, 1959). It is transmitted by aphids, and its host is limited to the genus *Tulipa* spp. and *Lilium* Spp. (De Kock *et al*., 2011). At present, approaches that have been used to detect TBV-infected tulips include visual observation, optical sensor detection, ELISA and molecular techniques. Since TBV interferes with the formation of anthocyanins, its symptoms are distinct in varieties that have red, purple and pink flowers. Therefore, part of TBV-infected tulips could be observed visually. However, it is not possible to identify color breaking in white- and yellow-flowering varieties. In addition, this approach needs to be performed during the short growing season which is quite labor consuming. To reduce the labor for screening TBV-infected plants, Polder *et al*., (2010) invented an optical sensor detection system that could be further used in autonomous identification and removal of TBV-infected plants during tulip production. This method showed a slightly larger error rate than visual assessment, and the test result was sensitive to cultivars and weather. Furthermore, this optical sensor system has not yet been commercialized. With the development of molecular biology, RT–PCR is becoming a popular method for virus detection (Ha *et al*., 2008). It has the benefits of good specificity, high sensitivity and rapid detection. However, this method is relatively expensive and cannot detect a large amount of samples at a time. At present, serological tests are preferred in large-scale virus detection. ELISA as a serological method is the most widely used approach to virus detection since it is sensitive, convenient, time-saving, and cost-effective (Shang *et al*., 2011). ELISA assessment is performed in the laboratory, allowing the testing of viruses in tulip bulbs during the storage period (Beijersbergen & van der Hulst, 1980). As early as 1966, antiserums against TBV were produced by Van Slogteren & de Vos (1966). However, these antiserums were inapplicable in ELISA test due to high background absorbance. Derks *et al*., (1982) optimized the purification procedure of tulip breaking virus and prepared new antiserums which can be used in ELISA. Dekker *et al*., (1993) isolated and characterized viruses causing color-breaking of tulip flowers by serology and PCR. The resistance of 263 tulip species and cultivars to TBV were evaluated by DAS-ELISA (Juodkaitė *et al*., 2012). Although research institutions in the Netherlands have detected TBV using ELISA for many years, the test kit has not been commercialized globally. Ordinary growers are still unable to carry out the ELISA test by themselves.

Using the purified virus as an immunogen to obtain antibody for ELISA is easily infected by the purification of virus and serological cross reactions among viruses (Shukla *et al*., 1992). Meanwhile, the isolation and purification of viruses is not easy. Many studies have attempted to express viral proteins in prokaryotic systems through genetic engineering and verified that this method can maintain certain immune activities (Kong *et al*., 2009; Liu *et al*., 2017; Wei *et al*., 2012). Up to now, few

fundamental studies have been carried out on TBVs. The currently known TBV genome (RefSeq: NC_043168.1) is 3.14 Kb, contains the genes *CI, 6K2, VPg, NIa-Pro*, and *Nib*. It encodes a polyprotein (GenBank: AHI04506) that consists of 1138 amino acids. Polyprotein is a chain of covalently conjoined proteins and is used by viruses to structure their proteome (Crépin *et al*., 2015; Yost & Marcotrigiano, 2013). Therefore, to obtain an ideal immunogen, cloning of the TBV polyprotein gene and prokaryotic expression of the protein were carried out in this study. Highly purified fusion protein was obtained by IPTG induction and used as an antigen to produce a monoclonal antibody against TBV. This study provides an important reference for the development of serological detection and lays the foundation for further study on the pathogenesis of TBV.

Material and Methods

Plant materials. TBV-infected tulip plants were collected and preserved in an isolation area of Qinghai University (Xining, China). Leaves of the tulip cultivar 'Barcelona' were sampled approximately 2 weeks after flowering. *Escherichia coli* BL21 (DE3), pMD™19-T Vector (Code No. 6013) and pCold™ I DNA vector (Code No. 3361) were provided by Takara Biomedical Technology (Beijing) Co., Ltd. *Escherichia coli* DH5α was provided by Sangon Biotech (Shanghai) Co., Ltd.

Gene clone and expression vector construction: According to the genome sequence of the tulip breaking virus isolate Texas Flame (NC_043168), primers of the *FK912_gp1* gene were designed using program Primer Premier 5.0. The specific forward primer was TBVF (5'-CGCGGATCCTCACGTCACATTGCGGATCA-3') and the reverse primer was TBVR (5'-CGGGGTACCGCACCCATGCCTCTAGTTGT-3'). The scribe lines are the restriction sites of *Bam*HI and *Kpn*I, respectively. Total RNA of leaves was isolated using the TaKaRa Universal RNA Extraction Kit (9767), and reverse transcription was carried out using the TaKaRa Prime Script™ II 1st Strand cDNA Synthesis Kit. The PCR mixture (25 μL) included 12.5 μL of 2×Taq PCR Master Mix (TIANGEN Biotech (Beijing) Co., Ltd.), 0.5 μL of cDNA (1000 ng/μL), and 1 μL of primer (10 mmol/L, both forward and reverse primer). The PCR procedure was set as follows: 94°C for 3 min; 30 cycles at 94°C for 30s, 55°C for 30 s, and 72°C for 1 min; 72°C for 5 min. The target fragment was purified and recovered using Tiangen's ordinary agarose gel DNA recovery kit (DP209). The purified PCR product was ligated into pMD19-T and then transformed into *E. coli* DH5α. White colonies were examined by PCR and positive colonies were sequenced by Sangon Biotech (Shanghai) Co., Ltd. Sequences were assembled by DNAStar and blasted using NCBI BLAST. The positive colonies were digested by the restriction enzymes *Bam*HI and *Kpn*I and then ligated to pCold™ I DNA vector (Code No. 3361) by T4 DNA ligase. The recombined plasmid was converted into *E. coli* DH5α competent cells and identified using bacterial liquid PCR and enzyme digestion.

Expression in *E. coli*: The recombination vector and empty pCold I vector (negative control) was transferred into *E. coli* BL21 (DE3). Transformant were cultured in Luria-Bertani (LB) medium containing kanamycin 50 ng/μL, at 37°C overnight. A single clone was cultivated in LB liquid medium until the OD_{600} of the bacteria solution reached 0.6. The inducer IPTG was added to the bacteria solution at 0.5 mM and cultivated at 20°C overnight. Bacterial liquid without IPTG was used as a negative control. Sediment of the bacterial liquid was collected and suspended in buffer A (PBS, pH 7.4) and dissolved thoroughly using a sonicator. After centrifugation, the sediment was dissolved using buffer B (including 8 M urea, 50 mM Tris-HCl and 300 mM NaCl, pH 8.0). Both the supernatant and sediment were prepared for SDS–PAGE (Berry *et al*., 2004).

Purification of recombinant protein: Buffer C (including 8 M urea, 50 mM Tris, 300 mM NaCl and 0.1% Triton X-100, pH 8.0) was used for cell suspension. The obtained cells were suspended in Buffer C and then disrupted by a sonicator. Crude protein was collected after centrifugation and then purified with a column of Ni-NTA agarose resin. The column was washed using a binding buffer (including 8 M urea, 50 mM Tris and 300 mM NaCl, pH 8.0) at a flow velocity of 5 ml/min. Incubation of the crude protein was carried out in balanced packing material for an hour. The incubated product was filled into the column and the fluent was collected. The column was balanced using binding buffer and washed with washing buffer (including 8 M urea, 50 mM Tris, 300 mM NaCl and 20/50 mM imidazole, pH 8.0). The column was then eluted using elution buffer (including 8 M urea, 50 mM Tris, 300 mM NaCl and 500 mM imidazole, pH 8.0). The purified recombinant protein was verified by SDS-PAGE and Western blotting. The verified protein was then dialyzed into protein preservation buffer, concentrated by PEG20000, filtered through a 0.22 μm membrane and preserved at -80°C.

Preparation of monoclonal antibody: The purified recombinant protein was used for antibody production in Balb/c mice. The experiment procedure was improved based on Zhang *et al*., (2019). Five mice were immunized five times over a 20-day interval. Mice were first immunized using a mixture of purified recombinant protein and Freund's complete adjuvant (v/v 1:1). The remaining four injections were given with a mixture of purified recombinant protein and incomplete Freund's adjuvant (v/v 1:1). After immunization, tail blood was collected for detection of valence by DAS-ELISA. Three weeks after the last injection, mice with the highest blood valence were selected for cell fusion. The spleen cells of the selected mouse were fused with myeloma SP2/0 cells. Monoclonal strains with high positive values were screened by subcloning. Monoclonal antibody was produced through the ascites method. Valence detection was carried out after purification of the monoclonal antibody. The negative control was serum (1:1000) from mice without immunization. The blank control was 5% skim milk powder. The ELISA plate was coated with the purified recombinant protein. Antiserum was used as the primary antibody after a series of multiple proportion dilutions from 1:1000 to 1:2048000. After incubation at 37 °C for 1 h, the coated plate was washed with

PBST (phosphate-buffered saline with 0.1% Tween 20). One hundred microliters of HRP-labeled donkey anti-mouse IgG (1:8000) was added to each well as the second antibody and incubated at 37°C for 45 minutes. The plate was then rinsed three times with PBST. An EL-TMB chromogenic reagent kit was used to verify the specificity of the antiserum. The optical density was determined by a microplate reader at 450 nm.

Results

Amplification of the target fragment and sequence analysis: A 470 bp gene fragment was amplified from TBV-infected tulip leaves (Fig. 1). The length of the fragment was the same as expected. The target gene was successfully inserted into vector pMD19-T. Sequence BLAST analysis showed that the inserted gene displayed 100% similarity with the sequence published in NCBI (GenBank: NC_043168), indicating that the obtained fragment was the correct TBV *FK912_gp1* gene and that no mutation was found.

Construction of the expression vector: The target gene was ligated with expression vector pCold I by T4 DNA ligase. The recombinant plasmid was sequenced and digested by the restriction enzymes *Bam*HI and *Kpn*I. The size of the digestion product was the same as expected (Fig. 2), indicating that the construction of the prokaryotic expression vector was successful. Sequencing results also indicated that the sequence was correct and could be used for further gene expression.

Expression and purification of the recombinant protein: A positive clone of the confirmed pCold I-TBV plasmid was transferred into *E. coli* BL21 (DE3). After expression, ultrasonication was carried out and SDS–PAGE electrophoresis was performed for both the supernatant and sediment. Results showed that the recombinant protein existed only in the sediment. A band of approximately 21 kDa was clearly observed (Fig. 3), which was consistent with the expected size of the protein (21.5 kDa). It was found that the recombinant protein was expressed mainly in inclusion bodies, which were then purified with a column of Ni-NTA agarose resin. A single band of approximately 21.5 kDa was observed (Fig. 4). Western blotting was carried out to further verify the purified fusion protein. A distinct band was observed at the expected position, suggesting it was the target protein (Fig. 5). The concentration of the fusion protein was 1.24 mg/ml and the purity was more than 90%.

Production of monoclonal antibody: The purified fusion protein was used as an antigen to produce antibody. After five immunizations, mouse 9009-2, which showed the highest blood valence (128K), was selected for cell fusion. Through cell fusion and subcloning, two hybridoma cell lines (2B12 and 4G6) with stable secretion of specific monoclonal antibodies against recombinant proteins were obtained. After the injection of BALb/c mice with 2B12 and 4G6, ascites was collected, purified and examined by ELISA with different consecutive dilutions (from 1:1000 to 1: 2048000). The valence of the monoclonal antibody was approximately 1:512000 (Fig. 6). This means that the pCold I-TBV-immunized mice had a good immunoreaction.

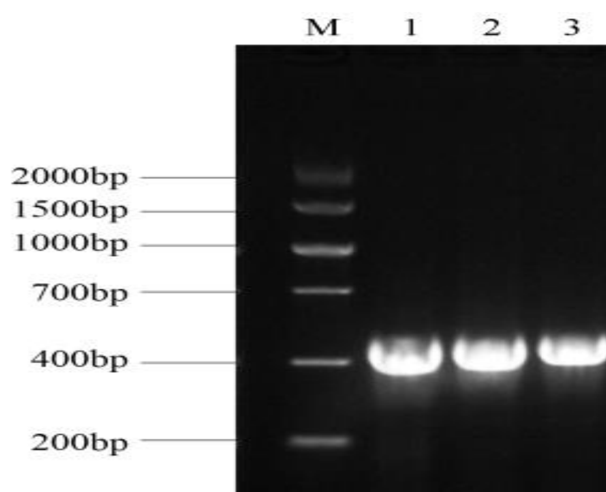

Fig. 1. RT–PCR products of TBV *FK912_gp1*. M represents DL2000 DNA Maker. Lanes 1-3 represent the band of the TBV *FK912_gp1* gene fragment.

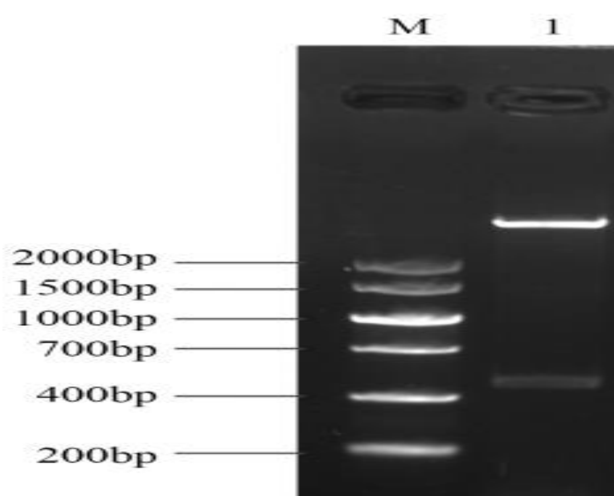

Fig. 2. Double restriction enzyme digestion of recombinant plasmid. M represents DL2000 DNA Maker. Lane 1 is the digestion product of pCold I by restriction enzymes *Bam*HI and *Kpn*I.

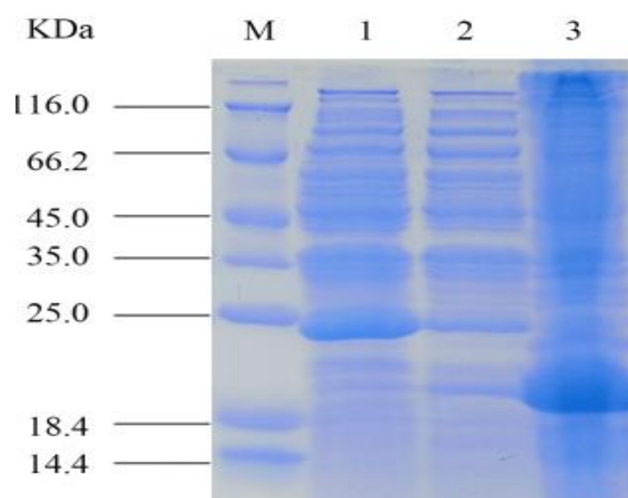

Fig. 3. SDS–PAGE result of the recombinant protein pCold I-TBV. M represents the protein molecular weight marker. Lane 1 is *E. coli* containing pCold I-TBV before induction. Lane 2 is the supernatant of induced *E. coli* transformed with pCold I-TBV. Lane 3 is the sediment of induced *E. coli* transformed with pCold I-TBV.

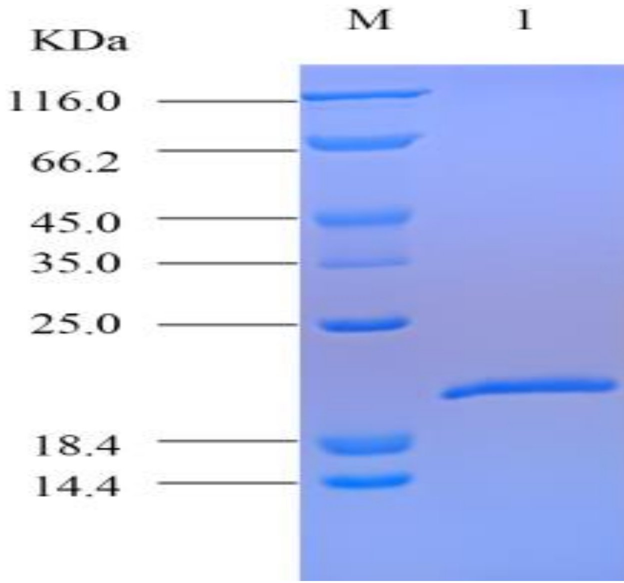

Fig. 4. SDS–PAGE analysis of purified pCold I-TBV protein. M represents the protein molecular weight marker. Lane 1 is the fusion protein.

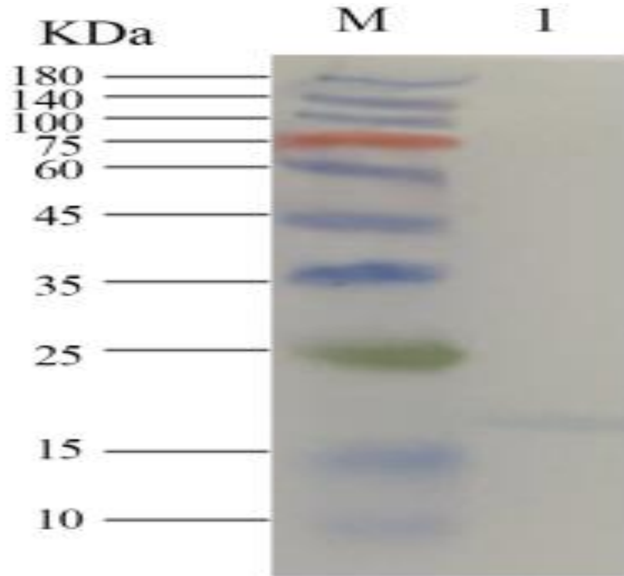

Fig. 5. Western blotting analysis of purified fusion protein. M represents the real band 3-color regular range protein marker. Lane 1 is purified fusion protein.

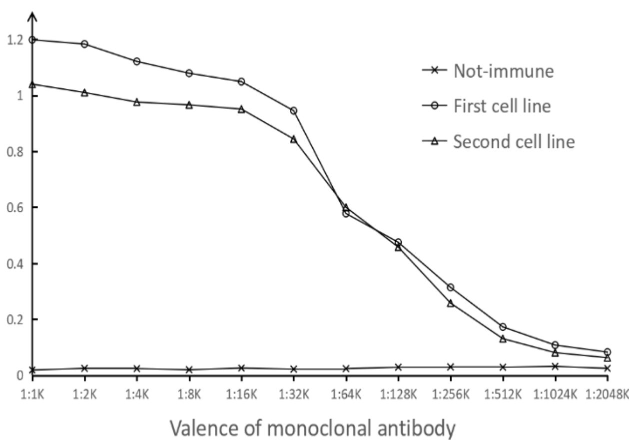

Fig. 6. Valence of the monoclonal antibody determined by ELISA.

Discussion

TBV is a devastating viral disease in tulip production. Since tulips are a vegetatively propagated crop, it is very important to pick out the infected bulbs before aphids start spreading the virus. At present, the detection of TBV mainly depends on visual observation during the growing season which is inefficient and labor consuming. Furthermore, the diagnosis of TBV-infected plants based on leaf and flower symptoms is unreliable (Van Tuyl & Creij, 2006). Thus, establishing a fast and reliable detection technique that can be carried out during the bulb storage period is particularly important to control the spreading of TBV. Serological assays are widely used in plant virus detection, in which ELISA test is sensitive, fast, and suitable for large-scale testing. However, the TBV test kit has not been commercialized globally, and the tulip grower cannot carry out an ELISA test themselves. In this study, a gene fragment of the TBV polyprotein was cloned and expressed in prokaryotic cells. The purified recombinant protein was used as antiserum to produce antibody. As a result, 2 lines of monoclonal antibodies were successfully obtained and could be further used in the detection of TBV.

Derks *et al*., (1982) purified TBV and produced antisera for use in ELISAs as early as 1982. However, the purification of potyvirus is quite difficult since virus paricles might aggregate together or aggregate with host plant. In addition, the antisera prepared against TBV presented strain specificity which will influence the effectiveness of using the antisera. Cloning and expression of target genes in *E. coli* is a common approach in the study of gene function, regulation of gene expression, production of diagnostic reagents and novel protein pharmaceuticals. The presentation of recombinant protein as an antigen to animals and the production of antibodies results in a relatively easy approach. The prepared antibody shows high efficiency and high sensitivity (Koolivand *et al*., 2016) (Xie *et al*., 2012). Lee & Chang (2008) produced antisera against orchid viruses using recombinant capsid proteins. The homemade antisera showed high sensitivity and specificity in ELISA tests.

An expression system using *E. coli* as a host is widely used in the production of recombinant proteins. The *E. coli* BL21 used in this study is a protease deficient strain (Dong *et al*., 2008). B strains of *E. coli* have a powerful secretory system and secrete minor proteases by themselves, making them very suitable for the expression of heterologous proteins (Yoon *et al*., 2009). Although *E. coli* has many advantages, it is not suitable for all genes. Some genes are difficult to express in *E. coli*, or the recombinant protein is not soluble (Assadi-Porter *et al*., 2008). It was confirmed that 20°C was the optimal temperature for the construction of the expression vector in this research. A high concentration of fusion protein was obtained after large-scale induction and cultivation. Insoluble protein did not occur, which indicated that the selected expression vector is suitable for the preparation of recombinant TBV protein.

Both monoclonal and polyclonal antisera could be used in ELISA tests. Compared with monoclonal antiserum, the specificity and accuracy of polyclonal antiserum is relatively low. Chung *et al*., (2009) produced both monoclonal and polyclonal antibodies against lily mottle virus (LMoV).

Polyclonal antibodies could not discriminate LMoV-infected lily plants and healthy plants. Monoclonal antibodies were more sensitive than polyclonal antibodies for the detection of LMoV in lilies. Monis *et al*., (2000) developed monoclonal antibodies that react to a grapvine leafroll-associated virus coat protein. They also found that polyclonal antiserum reacted not only to native viruses but also to heat-denatured viruses. At present, monoclonal antibodies are widely used in the detection of viruses, such as Tomato yellow leaf curl Thailand virus (Seepiban *et al*., 2017), Zucchini yellow mosaic virus (Chen *et al*., 2017), arabis mosaic virus (Li *et al*., 2011), citrus mosaic virus (Miyoshi *et al*., 2020), potato virus M (Yu *et al*., 2020), and Wheat dwarf virus (Zhang *et al*., 2018).

Conclusions

During this study, a 470 bp gene fragment was amplified from the TBV-infected tulip cultivar 'Barcelona' using RT–PCR. The target gene was inserted into vector pMD19-T and showed 100% similarity with the TBV *FK912_gp1* gene. It was successfully ligated with pCold I and then transferred into *E. coli* BL21 (DE3). It was proven that the recombinant protein existed only in sediment and was expressed mainly in inclusion bodies. The purified recombinant protein was further verified by Western blotting and used as an antigen for antibody production. After cell fusion and subcloning, two hybridoma cell lines (2B12 and 4G6) with stable secretion of specific monoclonal antibodies against recombinant proteins were obtained. The valence of the obtained monoclonal antibody was approximately 1:512000, which indicates that the pCold I-TBV immunized mice showed good immunoreaction. The obtained monoclonal antibody can be used in rapid serological detection of TBV. Meanwhile, results of this study also lay the foundation for further study on the function of the polyprotein.

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