EXPRESSION OF THE BARLEY YELLOW DWARF VIRUS-GAV MOVEMENT PROTEIN AND ITS DETECTION IN THE INFECTED AND TRANSGENIC PLANTS

J. XIE, X. WANG*, Y. LIU, Y. PENG, G. ZHOU

State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, West Yuan Ming Yuan Rd. 2, Beijing 100094, P.R. China

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Summary. – Movement proteins (MPs) that facilitate virus movement in the plants were identified in a number of plant viruses. In this study, full-length MP gene of the Chinese isolate Barley yellow dwarf virus-GAV (BYDV-GAV) was cloned and expressed in *Escherichia coli*. About 32% of the expressed MP was soluble providing the concentration of isopropyl-ß-D-galactopyranoside (IPTG), time of the induction, temperature and shaking speed were optimized. The soluble MP was purified using nickel-affinity column. Immune serum prepared against purified MP was used for the detection of MP in the BYDV-GAV infected leaves of oat and in the leaves of transgenic wheat plants expressing the full-length and truncated MP gene.

Key words: Barley yellow dwarf virus-GAV; movement protein; expression; immunization

Introduction

BYDVs are single-stranded positive-sense RNA viruses that are classified as the members of the family *Luteoviridae.* The most important viruses BYDV-PAV, BYDV-MAV and BYDV-PAS belong to the genus *Luteovirus*. (D'Arcy and Domier, 2005).

In China, wheat is the major crop that could be infected by BYDV. For many years, the crop losses have been observed in wheat grown in regions of northern and northwestern provinces of China that could reach 20–30%. The Chinese BYDV isolates were divided into four distinct groups: namely GAV, GPV, PAV, and RMV, respectively based on the aphid vector transmission phenotype. Some of them are not identical with those found in the United States (Zhou *et al*., 1987). For example, BYDV-MAV is transmitted only by *Sitobion avenae*, but Chinese isolate BYDV-GAV, which is serologically related to BYDV-MAV, is transmissible by *Schizaphis graminum* as well as *S. avenae* (Wang *et al*., 2001; Zhou *et al*., 1987).

BYDVs are naturally transmitted from the infected to healthy plants by at least 25 aphid species in a highly specific, circulative, non-propagative manner (Gray and Gildow, 2003). Viruliferous aphids inject the virus particles into the phloem tissues of healthy plants during the feeding process (Gildow, 1985; Gildow and Gray, 1993), where they replicate and move mainly within phloem tissues (Barker and Harrison, 1986; Derrick and Barker, 1997). The phloem limitation of the viruses is not due to their inability to replicate in cells other than phloem cells, but results from a failure of virus movement, which can be overcome by co-infection with another virus that invades all tissues of the plant (Barker, 1987; Young *et al*., 1991). It is generally accepted that cell-to-cell movement involves both virus-encoded MPs and host-encoded components (Atabekov and Taliansky, 1990). MPs have been identified in a number of plant viruses. It has also been shown that different MPs may facilitate cell-to-cell movement by different mechanisms (Leisner, 1999). The 17-kDa protein product of the ORF4

*Corresponding author. E-mail: xfwang@caas.net.cn; fax: +8610-62896114.

Abbreviations: BYDV = Barley yellow dwarf virus; GST = glutathione-S-transferase; IPTG = isopropyl-ß-D-galactopyranoside; MP(s) = movement protein(s); PLRV = Potato leafroll virus

plays a role of an MP in BYDV-PAV and Potato leafroll virus (PLRV) (Nass *et al*., 1998; Schmitz *et al*., 1997).

The most cost-effective and environmentally desirable form of disease control is the genetic resistance. However, natural resistance genes in wheat or barley to BYDVs are scant (Burnett *et al*., 1995; Jin *et al*., 1998). The relationship between the structure and function of MPs indicated that a transgenic plant expressing defective MP could block the function of viral MP during infection and the higher resistance to viral infection might arise (Cooper *et al*., 1995). Transgenic potato plants expressing a modified product of PLRV-ORF4 were produced and their resistance to the viral infection was verified. These plants were resistant to infection by homologous PLRV, as well as by Potato virus X and Y (Tacke *et al*., 1996). Transgenic plants that were not able to produce ORF-4 protein despite high transcription level, failed to exhibit virus resistance. In our laboratory, full-length and a truncated ORF4 gene of BYDV-GAV were transformed into wheat cultivar Yangmai 158 using biolistic particles. Primary resistance assays showed that the transgenic plants expressing full-length ORF4 were sensitive to the BYDV-GAV infection. On the other hand, transgenic plants expressing truncated ORF4 were resistant to viral infection in comparison with the control wheat cultivar Yangmai 158 (Jin, 2003).

It was difficult to purify MPs directly from the infected tissues due to their low concentration and transient expression. The expression of a fusion protein is a popular strategy widely used in the production of genetically engineered proteins (Terpe, 2003). However, it was difficult to obtain a soluble MP expressed in *E. coli*, because it was present as the inclusion bodies.

In this paper, we reported an improved procedure for high-level production of 17-kDa MP protein of the isolate BYDV-GAV. This protein was expressed in *E. coli*, purified by affinity chromatography, and used for the immunization of mice. Immune mouse serum against MP was used for the detection of MP in the infected leaves of oat and in the leaves of transgenic wheat with full-length and truncated MP gene.

Materials and Methods

Viruses and plants. The virus isolate BYDV-GAV was obtained in 1996 (Wang *et al*., 2001). The isolate has been maintained in oat plants and checked by vector transmission assay and ELISA (Rochow, 1969; Zhou *et al*., 1987). The oat – cultivar Coast Black and wheat – cultivar Yangmai 158 were used throughout this work. Transgenic wheat lines expressing the full-length and truncated ORF4 of BYDV-GAV were produced in our laboratory (Jin, 2003).

Molecular cloning of MP gene of BYDV-GAV. Total RNA was extracted from the infected leaves of oat plants, 2 weeks post inoculation with BYDV-GAV (Rochow, 1969; Zhou *et al*., 1987) using the Trizol reagent kit (Invitrogen, CA, USA). The cDNA was synthesized from total RNA using the Promega cDNA synthesis system with reverse primer of the capsid protein gene of BYDV-GAV (Wang *et al*., 2001). Full length of MP gene was obtained using primers MP1: 5'-GACATATGGCCCAAGGAGAGCAAGG-3' (*Nde*I restriction site is underlined), and MP2: 5'-CAGGATCCC GCCGAGCTCTCCCTGAATTC-3' (*Bam*HI restriction site is underlined). The PCR amplification was performed as followed: denaturation at 94°C for 5 mins, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. The final extension cycle was at 72°C for 10 mins.

Construction of expression plasmid pET21b-MP. The obtained MP amplicon was purified using a DNA gel extraction kit (Takara, Dalian) and cloned into the pGEM-T easy vector (Promega, Madison, WI). The ligated product was transformed into *E. coli* strain DH5α and positive clones were identified by PCR. Plasmid DNA was digested with *Nde*I and *Bam*HI and inserted into the His-tagged vector pET-21b (Novagen, Madison, WI), defined as pET21b-MP. Recombinant plasmid pET21b-MP was used to transform *E. coli* strain BL21 (DE3) pLysS (Novagen, Madison, WI). The positive clones expressing fusion MP were sequenced to verify the sequence of recombinant expression vector.

Optimization of expression conditions. To test the expression of the soluble MP fusion protein at the small-scale volume, positive clones of *E. coli* recombinants were grown in 5 ml of LB medium 1.0% peptone, 0.5% yeast extract, and 1.0% NaCl, pH 7.0 containing 100 μg/ml of ampicillin at 37°C for 8 to 12 hrs. The bacterial culture in volume of 500 μl was used for the inoculation of 10 ml fresh LB medium and incubated at 37°C until the A_{600} reached 0.6. Induction conditions as concentration of IPTG, time of induction, temperature and shaking speed were optimized. The concentration of 0.1, 0.2, 0.4, and 1.0 mmol/l of IPTG were tested and the samples were collected at 1, 2, 3, 4, 5, 6, 7, 8, 12 and 24 hrs after induction. To set the optimal temperature and shaking speed of the incubator, the cultivation was performed at the temperatures 37, 32, 28, 25, and 20°C, respectively, and speeds 220, 200, and 180 rpm, respectively.

To assess the total amount of expressed MP protein, a 1 ml of bacterial suspension was harvested by centrifugation at 13,000 x g for 20 mins at 4°C and the bacteria were mixed with 100 μl of loading buffer, analyzed by SDS-PAGE, 15% gel, and stained with Coomassie brilliant blue R250 (Sigma). To analyze expression of soluble MP, 5 ml of bacterial culture was harvested by centrifugation and suspended in 500 μl buffer A (50 mmol/l sodium phosphate buffer pH 7.4, 500 mmol/l NaCl), lysed by a sonication, and supernatants were analyzed by SDS-PAGE, 15% gel.

For the large-scale volume expression, the most productive clones identified in small-scale experiment were inoculated in 50 ml of LB medium for seed culture. After incubation overnight at 37°C, the seed culture was incubated in 1,000 ml of fresh medium and grown at 37°C. When A_{600} of the culture reached about 0.6, the conditions considered as optimal at the small-scale experiment were used: the concentration 0.4 mmol/l IPTG, temperature 28°C, time 8 hrs and 200 rpm shaking speed.

Purification of MP fusion protein. Purification of the His6-MP fusion protein was performed by His-Select™ HC nickel affinity gel (Sigma). Harvested bacteria were washed twice with 50 ml of buffer A, lysed by sonication, and centrifuged. The supernatant

and pellet were analyzed by SDS-PAGE, 15% gel. The supernatant was loaded onto a 10 ml affinity gel column pre-equilibrated with buffer A. To adjust the optimal concentration of imidazole for the elution of bacterial and MP fusion proteins, increasing concentrations 10, 20, 50, 100, 200, 300, 500 and 1000 mmol/l in 50 mmol/l sodium phosphate buffer pH 7.4 were used. Purified MP fusion protein was dialyzed against buffer A. Total protein was determined using Bradford protein detection kits (Sango Bio., Shanghai, China) with a modified method (Zor and Selinger, 1996).

Preparation of immune serum against MP fusion protein. Six BALB/c mice were immunized with 4 doses of 30 mg/dose of the purified MP fusion protein emulsified in Freund's complete adjuvant in 2-week interval. The mice were bled 2 weeks after the last inoculation. The titer of antiserum was determined using ELISA with 100 ng/well of purified MP protein as antigen (Jordan and Hammond, 1991).

SDS-PAGE and Western blotting. Leaves (0.2 g) of infected or transgenic plants were grinded in liquid nitrogen, mixed with 300 µl loading buffer and analyzed on SDS-PAGE, 15% gel. After PAGE, the gels were blotted onto nitrocellulose membranes (GE healthcare). Blots were incubated with the mouse anti-MP serum and bound antibodies were visualized with AP-conjugated anti-mouse IgG and stained with BCIP and NBT (Roche, Mannheim, Germany).

Results

Molecular cloning of MP gene and construction of expression plasmid

The full length of MP gene of BYDV-GAV contained 462 nts encoding a protein of 154 aa with calculated size 17.2 kDa. The complete MP gene of BYDV-GAV was obtained by RT-PCR using the primers MP1 and MP2. The primer MP2 was designed to delete the stop codon of MP gene and the two bases CG were added before *Bam*HI site in order to adjust the ORF to express the His6-tag of vector pET-21b. The obtained MP amplicon was digested with *Nde*I and *Bam*HI and cloned into a T7 promoter-driven fusion expression vector pET-21b to get pET21b-MP (Fig. 1). The expressed MP fusion protein contained 176 aa with calculated size of 19.7 kDa including a 6 aa His-tag and a 16 aa fusion tag of cloning site.

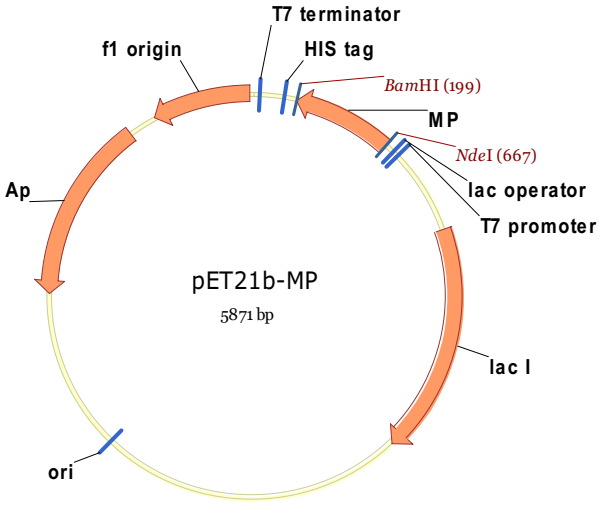

Fig. 1

Diagram of the recombinant expression plasmid pET21b-MP

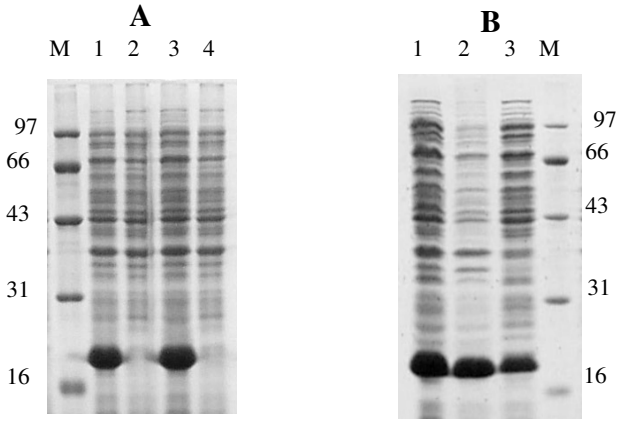

Fig. 2

SDS-PAGE analysis of expressed MP fusion protein in E.coli at the large scale

Panel A: productive clones after IPTG induction (lanes 1, 3), control empty clone with IPTG induction (lane 2), productive clone without IPTG induction (lane 4). Panel B: total bacterial lysate (lane 1), insoluble MP in pellet (lane 2), soluble MP in supernatant (lane 3). Molecular size markers (lane M).

Expression of MP fusion protein

Before expression of MP fusion protein at a large-scale volume, the optimal conditions for expression of the protein were determined. The optimal final concentration of IPTG for induction was 0.4 mmol/l and the induction time was about 8 hrs. The optimal temperature and shaking speed for induction of soluble MP were 28°C and 200 rpm, respectively. Selected *E. coli* clones inoculated in LB medium and induced with 0.4 mmol/l IPTG at 28°C and 200 rpm for 8 hrs, produced a protein with the estimated molecular size of 20 kDa (Fig. 2A, lane 1 and 3). This band was absent in the control empty clone (pET21b without MP gene) and in the positive clone without IPTG induction (Fig. 2A). The expressed soluble MP represented about 32% of the total expressed MP according to the densitometric determination of protein bands in SDS-PAGE (Fig. 2B).

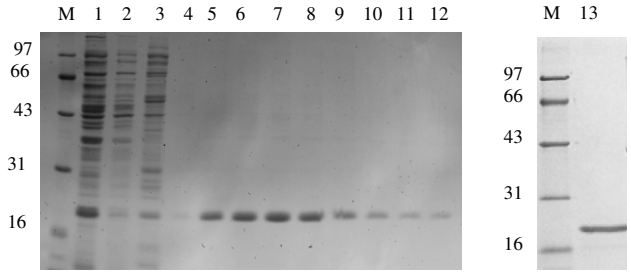

Fig. 3

SDS-PAGE analysis of the fractions collected at the purification of the MP fusion protein

Flow thru fraction (lane 1), fraction eluted by buffer A (lane 2); fraction eluted by buffer B (lane 3), fractions eluted by buffer C (lanes 4 to 12), purified MP fusion protein (lane 13), molecular size markers (lane M).

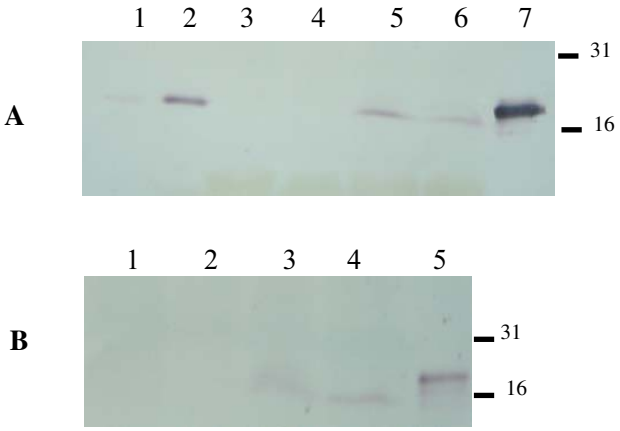

Fig. 4

Western blotting of BYDV-GAV-infected (A) and transgenic (B) plants

Panel A: 1 ng (lane 1), 10 ng (lane 2), 100 ng of purified MP (lane 7), non-infected oat (lane 3), non-infected wheat (lane 4), infected oat (lane 5), infected wheat (lane 6). Panel B: control wheat (lanes 1, 2), transgenic wheat with full length MP (lane 3), transgenic wheat with truncated MP (lane 4), 10 ng of purified MP (lane 5). Molecular size markers on the right.

Purification of MP fusion protein

The bacterial suspension in volume of 1,000 ml was used for purification. The bacteria were lysed to release the MP fusion protein and the supernatants were purified by the Ni-affinity column. The bound MP was eluted with buffer containing increasing concentration of imidazole ranging from 10 to 1000 mmol/l. The eluted samples were analyzed by SDS-PAGE and the imidazole concentrations 100 and 500 mmol/l were found as the most efficient for elution the bacterial proteins and the MP fusion protein, respectively.

For purification of the MP fusion protein, three buffers were used: 1) buffer A (see *Materials and Methods*) and 2) buffer B (50 mmol/l sodium phosphate pH 7.4, 100 mmol/l imidazole, 500 mmol/l NaCl) to elute the host cell proteins and 3) buffer C (50 mmol/l sodium phosphate pH 7.4, 500 mmol/l imidazole, 500 mmol/l NaCl) to elute the MP fusion protein (Fig. 3). The purified MP fusion protein was dialyzed against buffer A to remove imidazole. A total of 6 mg of purified soluble MP fusion protein at the concentration 0.28 mg/ml was obtained from 1000 ml of bacterial suspension (Fig. 3).

Preparation of antiserum against MP fusion protein

The BALB/c mice were immunized with the purified MP fusion protein. The titer of the antiserum was 1:10,000 determined by ELISA.

Detection of MP protein in infected and transgenic wheat plants

MP in the leaves of oat and wheat infected for 2 weeks by BYDV-GAV were detected by Western blotting. Purified MP fusion protein in amount of 1, 10, and 100 ng was used as the positive control and the healthy oat and wheat leaves were used as the negative control. MP fusion protein was detected by immune mouse serum prepared against MP protein. Bands with size 17 kDa and 19 kDa, respectively, were clearly detected in the infected leaves and in the samples of purified MP. Similar bands were not found in healthy oat and wheat leaves (Fig. 4A). Approximately 1.0 and 0.5 μg of MP were determined in 1 g of fresh infected oat and wheat leaves by densitometric analysis of bands in Western blotting.

Expressed MP was detected also in transgenic wheat expressing full-length or truncated MP gene with deletion of 19 aa from the C-terminus. Bands of 17 and 15 kDa, respectively, were detected in transgenic wheat with full length and truncated MP gene. However, they were not found in control wheat (Fig. 4B). These results confirmed that the MP genes were expressed in the transgenic wheat plants.

Discussion

The rapid increase in the information available on virus movement has come by the application of a variety of methods ranging from cytological to molecular techniques. These include classic methods, protein expression, tissue blotting, electron microscopy, viral genome tagging and mutagenesis, microinjection, and transgenic plants expressing MPs (Hull, 2002). The properties of viral MPs have been studied on the gene(s) expressed in *E. coli* or in

insect cells (Brill *et al*., 2000). Molecular techniques such as Western blotting and tissue blotting are used to identify the stages and tissues in which MPs are expressed (Nass *et al*., 1998; Schmitz *et al*., 1997). Transgenic plants expressing a modified MP gene must be identified by Western blotting, because transgenic plants, which did not express MPs despite high transcript levels, failed to exhibit virus resistance (Tacke *et al*., 1996).

Tobacco mosaic virus MP expressed in *E. coli* accumulates in the inclusion bodies that might contain single-stranded RNA. The inclusion bodies can be solubilized in buffers containing urea, but after removal of the urea, aggregation recurs creating precipitates that contain protein/RNA complexes (Brill *et al*., 2000). Published data showed also that the solubility of Tobacco etch virus (TEV) protease overexpressed in *E. coli* was extremely low (Kapust and Waugh, 2000; Lucast *et al*., 2001). The expression the MP gene of BYDV and PLRV in *E. coli* was not reported in details (Nass *et al*., 1998; Schmitz *et al*., 1997). In our previous study, GST-tagged form of full-length BYDV-GAV MP was expressed in *E. coli*, but the majority of the synthetized MP remained in the insoluble fraction after the cell lysis. The preparation of an active MP in a high concentration should undergo troublesome denaturation and renaturation process. In addition, the antiserum against GST-MP fusion protein showed high nonspecific reaction with plant proteins. GST-MP fusion protein used as an antigen for immunization of animals produced antibodies that were mostly directed against GST tag protein and not against MP protein. Maybe the reason was an inappropriate relation in molecular sizes of GST tag (26 kDa) and full length MP (17 kDa) (unpublished data).

In the present study, we used a T7 driven expression plasmid pET-21b to express His-tag fused MP protein. The His-tag presented only about 2 kDa, what might improve the specificity of the prepared antiserum. T7 system is well-known for its high expression level of the heterologous protein, mainly due to strong transcription initiation activity of T7 promoter and T7 RNA polymerase. The enhanced mRNA level improved the expression of target protein, but also led to the formation of inclusion bodies due to incorrect folding caused by high translation speed and incorrect hydrophobic interaction of nascent peptides. In order to obtain sufficient amount of the soluble fusion MP, different concentrations of IPTG, induced temperatures and times of cultivation, as well as shaking speeds were analyzed. Finally, about 32% of expressed MP was soluble under the optimal expression conditions that were achieved at the low concentration of IPTG, low temperature, and short cultivation time. Among these conditions, cultivation temperature was the most important factor. Immune serum against MP prepared with purified soluble MP could detect MPs evidently in infected leaves of oat and in the leaves of transgenic wheat with full length and truncated MP gene.

In this study, two-third of the total expressed MP was still present in the insoluble inclusion bodies under the optimal conditions. Even after purification, the purified MP has a low solubility in buffers without detergents. We found that the solubility of purified MP was about 0.3 mg/ml in buffer A, but when the concentration of NaCl in the buffer was below 200 mmol/l, the solubility of MP decreased rapidly. The poor solubility of MP might be one of its characteristics, parallel to binding to nucleic acids, targeting to and modification of the size exclusion limit of plasmodesmata, and transport of the viral genomes through the intercellular connections (Leisner, 1999). MP behaves also as an intrinsic membrane protein, promotes the formation of the endoplasmic reticulum (Brill *et al*., 2000). Those characteristics required a high lipid-compatibility of MP.

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