Cloning and expression of the coat protein gene of *Barley yellow dwarf virus*-PAV in *Escherichia coli*

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Abstract
Due to the restriction of Barley yellow dwarf virus (BYDV)-PAV particles to the phloem tissue and very low virus titers, purification of the virus is difficult. The aim of this study was to prepare antibody against viral coat protein without purifying the virus. To produce recombinant coat protein, the coding sequence was first amplified from a PAV full-length cDNA clone by polymerase chain reaction (PCR), ligated into a vector (pBluescript SK+) to check the sequence, and subcloned into an expression vector (pGEX-2T). It was then transformed into Escherichia coli DH5α by electroporation. The open reading frame 3 (ORF3) was linked in-frame to the gene encoding glutathione-S-transferase (GST; 26 kDa) and expression induced by IPTG. The expressed coat protein was purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for use as an immunogen. The antisera to BYDV-PAV recombinant coat protein reacted in Western blot analysis with partially purified BYDV-PAV. These antisera were also used to detect BYDV-PAV by immunogold electron microscopy of thin section of barley tissues. The results indicated that BYDV-PAV coat protein can be produced in high yields by E. coli, which provides the ability of simple purification, and because of proper antigencity, can be exploited for diagnostic applications.
Keywords: *Barley yellow dwarf virus, Expression vector, Polyclonal antibodies, E. coli, Coat protein, PAV.*

INTRODUCTION

Barley yellow dwarf virus (BYDV)-PAV is a species of the genus *Luteovirus* in the family *Luteoviridae* (Van Regenmortel *et al.,* 2000). The genus *Luteovirus* is a very important genus of plant viruses, which infects a wide range of agronomically important plants. The virus particles of the genus *Luteovirus* are small isometric particles of 25-30 nm in diameter. Luteoviruses are not mechanically transmissible and transmission occurs only by aphids. The genome of luteoviruses consists of a single-stranded positive-sense RNA molecule that is 5.5-6 kb in size and contains six open reading frames (ORFs); ORF1 and ORF2 encode for putative replicase proteins, ORF3 encodes a 22 kDa coat protein (CP), ORF4 is thought to be involved in virus movement in the plant, the products of ORF5 is necessary for aphid transmission, while the function of ORF6 is presently unknown (Hull, 2002; Miller *et al.,* 1995; Mayo and Ziegler-Graff, 1996; Smith and Barker, 1999). The genomic RNA of these viruses contain neither cap-structure nor genome-linked protein (VPg), and is non-polyadenylated (Shams-bakhsh and Symons, 1997; Miller and Rasochova, 1997). The virus is mainly restricted to the phloem tissue of the host plant, and thus accumulate in very low concentrations and purification of these virus is therefore very difficult.

Since 1970s several variations of the serological techniques have been used widely by pathologists and have increased tremendously the ability of plant pathologists to detect and study plant viruses. Now, the applications of plant virus serology are numerous. It is used to determine relationships between viruses, to identity a virus causing a plant disease, to detect virus in foundation stocks of plants as well as to detect symptomless virus infections. It can also be used to measure virus quantitatively, and to locate the virus within a cell or tissue (Hull, 2002 and Agrios, 1997).

The main problems with some plant viruses such

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as those of genus *Luteovirus* are the often difficult and tedious procedures to purity of purified virus particles from infected plants, which is used to prepare antibodies. To overcome this problem, we tested whether polyclonal antibodies prepared to the BYDV-PAV coat protein gene expressed in *E. coli* would be useful in developing inexpensive and simple immunological reagents for detection of BYDV-PAV.

MATERIALS AND METHODS

Virus, bacterial strain and plasmids: The PAV isolate of BYDV collected from a field near Adelaide, South Australia and kindly supplied by Monique Henry, University of Adelaide. *E. coli* strain DH5α (Stratagene, USA) was used for cloning while *E. coil* strains M15, JM105 and DH5α were used as hosts for the expression vector. The pBluescript SK(+) plasmid (Stratagene, USA) was used for routine cloning and pGEX-2T plasmid was used as an expression vector.

Virus purification: BYDV-PAV was purified from infected oat plants (*Avena sativa* L. cv New Zealand Cape) by modification of the methods described by Hammond *et al.* (1983) and D'Arcy *et al.* (1989).

Oligonucleotide primers: Oligonucleotides for BYDV-PAV-CP amplification were designed according to the sequence published by Miller *et al.* (1988), amplifying a 616 bp fragment. Primers PAV-2857 (5′ATGGATCCAATTCAGTAGGTCGTAGA3′) consisted of 26 nucleotides complementary to the 5′-terminal nucleotide sequence of ORF3 with a *Bam*HI restriction site (underlined) at the 5′-end and PAV-3459 (5′ATGGATCCCTATTTGGCCGTCATCAA3′) consisted of 26 nucleotides the 3′-terminal sequence of ORF3 with a *Bam*HI restriction site (underlined) at the 5′-end.

Polymerase chain reaction (PCR): PCR was performed in a 20 µl reaction mixture containing one unit of Vent DNA polymerase (New England Biolabs, USA). Reaction was carried out using recommended buffer conditions (10 mM KCl, 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% (v/v) Triton X-100), except that the concentrations of DNA primers and Mg^{2+} ions (present as $MgSO_4$) were optimized for each reaction. PCR reactions utilizing Vent DNA polymerase contained dNTPs at a concentration of 500 µM each. The mixtures were incubated at 94°C for 30 seconds, followed by 35 amplification cycles of 94°C for 5 seconds, 60°C for 5 seconds, and 72°C for 5 seconds each. PCR reaction was carried out on automated machines (DNA Thermal Sequencer) supplied by Corbett Research (Australia).

Cloning: Restricted vector (20-50 ng) was ligated with the DNA fragment to be cloned in molar ratios 3:1, 2:1 and 1:1 (vector:insert) respectively. The ligation was carried out in a volume of 20 µl containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10mM dithiothreitol, 0.5 mM ATP and T4 DNA ligase (Bresatec, Australia). One fifth of a unit of T4 DNA ligase was used for sticky-end ligations, or one unit for blunt-end ligations. The mixture was incubated either at room temperature for 4h or at 4°C overnight. The ligated material was used for the transformation of *E. coli* according to the standard protocol (Sambrook *et al.,* 1989).

DNA sequencing: To make sure that the amplification and construction processes did not affect the base sequence of BYDV-PAV coat protein gene; the dideoxynucleotide chain termination method (Sanger *et al*., 1980) was used to determine DNA sequence of pBS-P3.

Expression and purification of the recombinant BYDV-PAV coat protein: An overnight culture of *E. coli* DH5α cells containing the pGST-2TP3 construct was diluted 10 times into 20 ml of Luria-Bertain (LB) medium containing ampicilin (100 mg/ml) and incubated at 37°C for 1h before adding isopropyl-β–D thiogalactopyranoside (IPTG) to 0.2 mM. After further incubation for 3h and centrifuged, cell pellets were resuspended in SDS-loading buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% SDS) and proteins separated by 12% SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). After staining with Coomassie brilliant blue R250 (0.25% w/v in 40% methanol and 10% acetic acid) and destained in 12% methanol and 7% acetic acid, a band corresponding to the GST-P3 fusion protein was excised from the gel. The gel slice was cut into small pieces and the GST-P3 fusion protein was eluted electrophoretically in a BIOTRAP electroeluter (Schleicher and Schuell Inc.), according to the manufacturer's instruction.

Antibody preparation: Antibody was prepared against purified recombinant GST-P3 fusion protein (100 µg) in SDS loading buffer, emulsified with an equal volume of complete Freund's adjuvant (Sigma,

USA) and injected subcutaneously into a rabbit; followed by two booster injections (100 μg) of protein in SDS loading buffer by mixing an equal volume of incomplete Freund's adjuvant (Sigma, USA) at two weekly intervals. Antiserum was collected two weeks after the last booster injection.

Western immunobloting assay: The proteins of the partially purified BYDV-PAV particles were extracted in an equal volume of SDS gel-loading buffer, heated at 95°C for 5 min and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) system (4% stacking gel, 12% separation gel). Proteins were transferred to a nitrocellulose membrane (0.2 μm, Schleicher and Schuell) using a semi-dry transfer cell (Bio-Rad) according to the instructions of the manufacturer. Proteins were detected using polyclonal antiserum raised against the GST-P3 fusion protein. Blots were developed using alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega, USA) as described by Blake *et al.* (1984).

Transmission electron microscopy and immunolabeling: After infestation, samples were taken daily for 10 days from the midrib and stem of the primary leaf of healthy and BYDV-PAV infected barley plants. Samples were cut into small pieces of approximately 1 mm^2 and were then fixed in a mixture of 3.5% paraformaldehyde and 0.5% glutaraldehyde in 50 mM pipes buffer, pH 7.0, at 4°C overnight on a rotator. Following the fixation, the samples were washed and dehydrated in a graded ethanol series, and were then infiltrated with and embedded in LR Gold. Samples were allowed to polymerize. Ultra-thin sections were cut with a "LKB glass knife" on a "Reichert-Jung Ultracut-E" ultramicrotome. Silver sections were transferred onto coated G200 nickel grids. The grids were coated with 0.8% pioloform as described by Hayat (1989). Ultra-thin sections were blocked with 2.5% bovine serum albumin (BSA), 10% normal goat serum and 10% skimmed milk. Grids were incubated with one of two sera i.e., the polyclonal antiserum raised against the GST-P3 fusion protein and preimmune rabbit serum. After thorough washing, grids that were incubated with the polyclonal antiserum or normal rabbit serum were incubated with goat anti-rabbit IgG-gold (15 nm) conjugate (Amersham, England). Following washing, sections were stained with 4% uranyl acetate pH 4.0 and 0.3% $KMnO_4$ for 15 min, and then washed with water. Sections were viewed in a Philips CM 100 electron microscope.

RESULTS

ORF3 of BYDV-PAV was amplified by PCR from a full-length cDNA clone of BYDV-PAV (Young *et al.,* 1991) using primers PAV-2857 and PAV-3459. The amplified BYDV-PAV coat protein gene was purified by agarose gel electrophoresis and ligated into the *Sma*I site of the pBluescript SK+ vector and used to transform *E. coli* DH5α to check the sequence. The identity of the clone and the fidelity of the PCR product were verified by dideoxy chain termination sequence analysis. The resulting cDNA clone designated pBS-P3, was used to generate constructs for the expression of the coat protein product of the ORF3 in *E. coli.*

The cDNA clone pBS-P3 was digested with the restriction enzymes *Sal*I and *Eco*RI, and the recessed 3′-ends were filed using the Klenow fragment of *E. coli* DNA polymerase I. The reaction mixture was electrophoresed on a 1% agarose gel. A 356 bp DNA fragment was purified and ligated into the *Sma*I site of an expression vector (pGEX-2T) and transformed into *E. coli* DH5α by electroporation. The orientation of the insert in pGEX-2T was determined by restriction analysis and the recombinant plasmid was designated pGEX-2TP3. The ORF3 was linked in-frame to the gene encoding glutathione S-transferase i.e., GST having 26 kDa).

It was initially attempted to express the entire coat protein of BYDV-PAV. Consequently, the full-length ORF3 cDNA was cloned into the *Bam*HI site of the bacterial expression vector pGEX-2T and transformed into *E. coli* strain DH5α. A variety of expression conditions were examined. Post-induction incubation temperature (27°C and 37°C), final IPTG concentration (0.2 mM and 1 mM), media (LB, 2YT, and FTB), host strain (M15, DH5α and JM105) and length of incubation following induction (1, 2, and 3 h) were the varied parameters. None of the conditions used, however, gave rise to sufficient levels of protein expression for detection in SDS-PAGE. Western immunoblot analysis of the proteins extracted from induced and non-induced transformed cells using a polyclonal antiserum against GST protein also failed to detect the expected fusion protein. These results suggested that at least a part of the recombinant ORF3 protein is toxic to *E. coli* cells. As a result, it was decided to express different parts of the coat protein in bacterial cells. A 489 bp *Bam*HI/*Eco*RI DNA fragment from the pBS-P3, which contained approximately 80% of ORF3 was cloned into the pGEX-2T and transformed into the *E. coli* strain DH5α. After inducing of the transformed

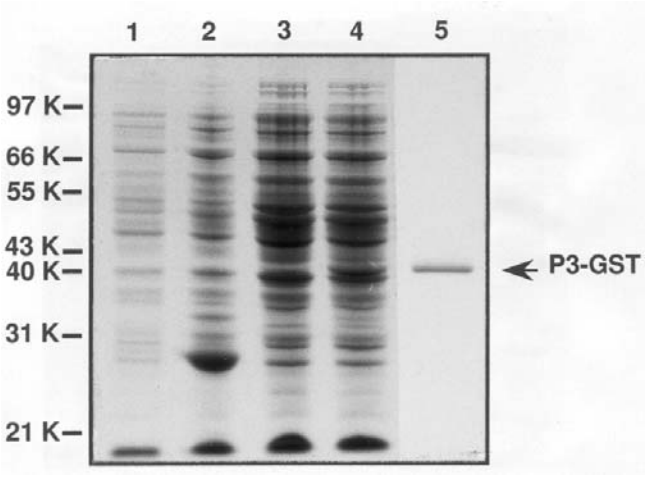

Figure 1. Expression and purification of the GST-P3 fusion protein. Expression of the GST-P3 fusion protein in *E. coli* cell line DH5α bacterial culture of pGES-2TP3 and pGEX-2T vector transformants grown and induced with IPTG at 0.2 mM final concentration for 3h prior to lysis. The total protein extract prepared from the cultures were electrophoresed on a 12% SDS-polyacrylamide gel and stained with 0.2% Coomassie blue. Lane 1, pGEX-2T non-induced, Lane 2, pGEX-2T induced, Lane 3, pGEX-2TP3 non-induced,Lane 4, pGEX-2TP3 induced, Lane 5, purified GST-P3 fusion protein.

cells with IPTG, the expected protein was not expressed to a level detectable on a SDS-PAGE or by Western immunoblot analysis.

Finally, the pBS-P3 was digested with *Sal*I and *Eco*RI and the resulting 356 bp DNA fragments, which contained the middle part of ORF3, was cloned into the pGEX-2T vector and transformed into the *E. coli* strain DH5α. Expression of this GST-P3 fusion protein was successful. The total bacterial protein extract was electrophoresed on a 12% SDS-PAGE gel; a 40 kDa (GST, 26 kDa , P3, 14 kDa) recombinant protein was detected in the induced control but was absent in the non-induced control (Fig. 1). The specificity of the recombinant protein was confirmed by Western immunoblot analysis using two antisera: the antiserum against the GST domain of the fusion protein and the antiserum against the BYDV-PAV particles (data not shown).

Particles of BYDV-PAV were detected seven days after inoculations in an ultra-thin section of infected barley by immunogold labeling (Fig. 2a) while the virus particles were not identified in the control (non-infected) samples (Fig. 2b).

DISCUSSION

Three different cDNA clones of BYDV-PAV ORF3 were constructed. Only the GST-P3, which contains the middle part of the coat protein (14 kDa of the total 22 kDa), was successfully expressed in *E. coli* fused to GST. This may be because the N-terminal sequence of the coat protein is toxic to the bacterial cells.

Polyclonal antisera prepared against BYDV-PAV coat protein expressed in *E. coli* served as a useful serological probe for BYDV-PAV in Western blots and immunogold labeling in electron microscopy. BYDV-PAV coat protein was readily detected in Western blots of the partially purified BYDV-PAV from infected

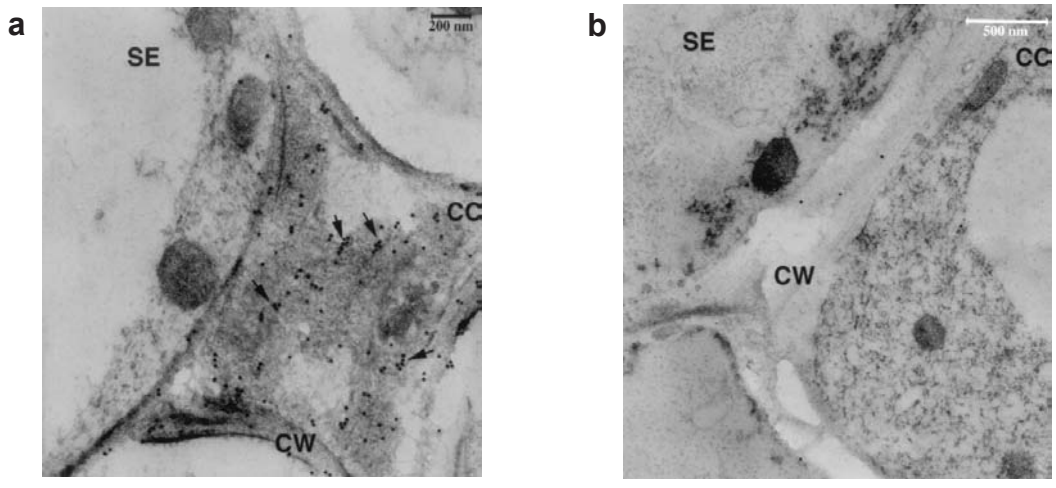

Figure 2. Immunogold localization of BYDV-PAV virus particles in barley leaf , 7 days after inoculation: infected plant (a), non-infected plant as a control (b). Thin sections were treated first with the antiserum against GST-P3 fusion protein and then with a goat anti-rabbit immunoglobulin conjugated to 15 nm of gold particles (Amersham, England). Arrows show gold particles. Abbreviations: companion cells (CC), sieve elements (SE), cell wall (CW).

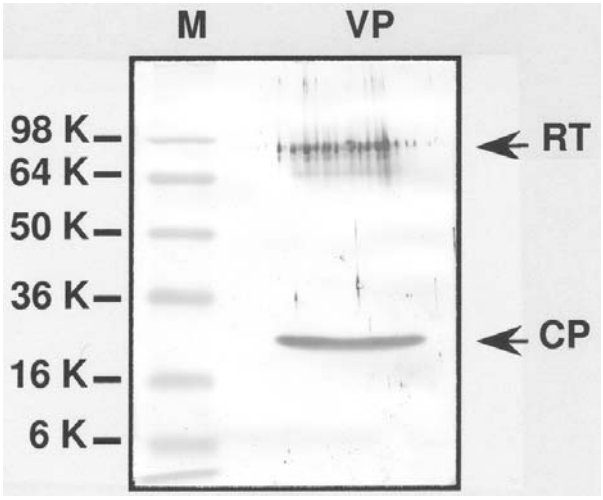

Figure 3. Western immunoblot analysis of partially purified BYDV-PAV virions. Partially purified BYDV-PAV virions were electrophoresed on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Western immunoblot analysis was performed with antisera raised against GST-P3 fusion protein as the primary antibody and alkaline phosphatase conjugated anti-rabbit secondary antibody. The antibody antigen complex was detected using a color reaction with BCIP/NBT substrates. CP, coat protein; RT, readthrough protein.

plants, with polyclonal antiserum raised against the GST-P3 fusion protein (Fig. 3). The polyclonal antiserum raised against ORF3 detected two proteins: one with an apparent molecular mass of about Mr 72 kDa and one Mr 22 kDa protein, indicating that in addition to coat protein (22 kDa), virion of BYDV-PAV contains another protein (72 kDa) which contains ORF3 sequences. This is not unusual, as read-through protein associated with virions have been detected in the genus *Luteovirirus* (Shams-bakhsh and Symons 1998; Wang *et al.,* 1995).

Virus particles were detected in ultra-thin sections of barley plants by immunogold labeling (Fig. 2). Addition of 1% skimmed milk to the blocking buffer significantly reduced the level of non-specific labeling in uninfected samples. However, very few gold particles were still observed attached to the membrane of organelles and cell walls of uninfected barley. This is commonly observed in many plant tissues (Nass *et al.,* 1995). Immunogold labeling of BYDV-PAV particles in infected barley plants indicated gold particles to be mainly distributed in the cytoplasm of the companion cells and also at a lower level in the cytoplasm of the sieve elements but not in other cell types. These results confirm observations made in earlier studies (Gill and Chong, 1975, 1976, 1979) that the particles of BYDV-PAV are restricted to the sieve element and companion cells of the phloem tissue.

The results of this work, together with those for *Potato leafroll virus* (PLRV) (Shepardson *et al*., 1980), provide evidence to suggest that virus particles of BYDV-PAV are detected first in the cytoplasm of infected phloem cells. In contrast, it has been shown that the virus particles of *Beet western yellows virus* (BWYV) (Esau and Hoefert, 1972) and *Cereal yellow dwarf virus* (CYDV)-RPV (Gill and Chong, 1976) are seen first in the nuclei of infected phloem cells. These results might be due to the difference in RNA-dependent RNA polymerases in these viruses.

In this article, we have produced polyclonal antibodies against BYDV-PAV recombinant coat protein expressed in *E. coli.* These serological probes are relatively inexpensive to produce (Vaira *et al*. 1996), are useful for the detection of BYDV-PAV, and negate the need to obtain, propagate, and purify virions for use as immunogen. This is very important in the viruses such as the genus *Luteovirus*, which are very difficult to purify.

Acknowledgment

We would like to thank Dr. MJ Young for providing the full-length cDNA clone of BYDV-PAV, Dr. Monique Henry for supplying BYDV-PAV, Dr. J. Randles for providing antiserum against BYDV-PAV, and Dr. Neil Shirly for providing the oligonucleotides.

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