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Over expression of Recombinant CP3 Protein of Rice Tungro Spherical Virus in Prokaryotic Expression System

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ABSTRACT

Currently, serological based diagnosis of rice tungro disease (RTD) is being limited by the availability of antisera. This study aims to produce recombinant protein from the coat protein (CP)3 of rice tungro spherical virus (RTSV) as a potential antigen in producing high titre antibodies for use in detection of RTD. Amplified RTSV CP3 gene of approximately 900 bp was cloned into pETSUMO vector. The recombinant plasmid was transformed into cloning host Mach1™-T1® *E.coli* cells and screened for recombinant gene in the correct orientation. Plasmid DNA from positive transformants was isolated and transformed into expression host BL21 (DE3) *E.coli* cells. One positive clone was selected for large scale expression where the expressed protein was purified and analysed by SDS-PAGE and Western blot. This recombinant RTSV CP3 protein, 46 kDa in molecular mass with 6x His-tag and SUMO protein fused to its N-terminal, was found to be antigenic when it reacted with our in-house generated polyclonal antibodies against tungro viruses.

INTRODUCTION

Rice tungro spherical virus (RTSV) is a plant picornavirus from the family *Sequiviridae* and the genus of *Waikavirus.* It assists in the transmission of rice tungro bacilliform virus (RTBV), the major causative agent of expression of rice tungro disease (RTD) symptoms, by green leafhopper, *Nephotettix* sp. [1-3]. Rice plants infected by RTSV alone are either asymptomatic or showed mild growth reduction [4].

RTSV has a positive-sense single-stranded, polyadenylated RNA genome of approximately 12 kb contained within a capsid composed of three coat proteins (CP) [5]. The genome encodes a single large polyprotein of 3473 amino acids which included the three CPs namely CP1, CP2 and CP3. The molecular mass of CP1, CP2 and CP3 are estimated to be 22.5 kDa, 22.0 kDa and 33 kDa respectively. Among the three CPs, CP3 is probably the major determinant on the virus surface [6]. Currently the most common method used for the diagnosis of RTD is by visual observation, however the results can be unreliable and not specific.

Another common diagnosis is by using molecular methods however these techniques can be costly especially for screening large amount of samples [7-10]. Used of serological methods had also been reported such as enzyme-linked immunosorbent assay (ELISA) and latex flocculation test [11-13]. Although the serological methods has been shown to be more reliable and cost effective, the availability of high titre antisera against tungro viruses for use in serological identification of RTD is limited by low concentration of tungro viruses in plants for antibody production [7, 14].

Therefore for this study we were interested to evaluate the potential of using recombinant antigen as an alternative to generate high titre antibody for to be used in a serological detection of RTD. This study was focused on the CP3 glycoprotein which is reported to be the major determinant on the virus surface. Here we had successfully cloned and expressed the CP3 of RTSV as a recombinant fusion protein to 6X histidine for ease of purification in a prokaryotic expression system.

MATERIALS AND METHODS

Tungro isolate

A tungro isolate from Sabah, Malaysia provided by the Department of Agriculture, Sabah, Malaysia was used for this project. This isolate is maintained as RTSV and RTBV in rice cultivar, *Taichung Native* 1 (TN1) in plant houses in the Agriculture Research Centre (ARC) at Tuaran, Sabah, Malaysia.

RNA extraction

Infected TN1 leaves were grounded with liquid nitrogen using pestle and mortar. The nucleic acids of RTSV was extracted from homogenized infected TN1 leaves in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) using High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's instructions.

First strand cDNA synthesis

First strand cDNA of RTSV CP3 was synthesised from extracted nucleic acids using MuLV reverse transcriptase (Fermentas). Reverse transcription was carried out at 37 °C for 60 min in a thermal cycler (MJ Research PTC-100, Canada). The reaction mixture prepared in a 0.2 ml tube contained 5.5 µl of extracted total nucleic acids, 1.0 µl of 20 pmol of RTSV CP3 reverse primer (Table 1), 2.0 µl of 5X RT buffer, 0.5 µl of 10 mM dNTPs mix and 1.0 µl of M-MuLV reverse transcriptase (20 unit /µl).

Polymerase chain reaction

Template from first strand cDNA were added with 5 µl of 10X PCR buffer, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP mix, 1 µl of both RTSV CP3 forward and reverse primers (Table 1) at 20 pmol/µl, 0.5 µl of *Taq* DNA polymerase (5 unit/µl) (Fermentas) and sterile ultra high quality (UHQ) water added to make a final volume of 50 µl. Amplification was carried out using the following program: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min and extension at 72 °C for 1 min, final extension at 72 °C for 7 min and hold at 4 °C.

Cloning into pETSUMO vector

Amplified PCR products were resolved on a 1.5% agarose gel by electrophoresis and analyses using a gel imager BioRad Gel Imaging System). Expected DNA fragment containing the CP3 gene was excised from the gel and purified using QIA quick PCR Purification Kit (Qiagen, Germany) according to manufacturer's instructions. Purified PCR products were ligated into pETSUMO vector (Champion™ pETSUMO System, Invitrogen, Carlsbad, CA, USA) in a molar ratio of 1:2 (vector: insert) and incubated overnight at 16 °C. The ligation mixture prepared consisted of 1 µl of 10x ligation buffer, 2 µl of pETSUMO vector (25 ng/ ml) , 1 µl of T4 DNA (4.0 Weiss unit), 4 µl of template and sterile UHQ water added to make a final volume of 10 µl. Another ligation mixture of pETSUMO vector without insert was prepared.

Table 1: Primers used in cloning of RTSV CP3 gene.

Primer	Sequence	Source or reference
RTSV CP3 forward	5'- GAC TTT GGA AGA AGC CTA -3'	*Genbank accession no. U70989*
RTSV CP3 reverse	5'- TTG AGG ATC TGA CAC CGT -3'	*Genbank accession no. U70989*
SUMO forward	5'- AGA TTC TTG TAC GAC GGT ATT AG-3'	Invitrogen

Transformation into cloning host

Two microliters of ligation mixture was then transformed into One Shot® Mach1™-T1® *E. coli* cells (Invitrogen, Carlsbad, CA, USA). The mixture was incubated on ice for 30 min, subjected to heat-shock at 42 °C for 30 sec and finally cooled on ice for 2 min. Transformed cells were recovered by adding 250 µl of S.O.C medium (2% tryptrone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) into the tube and incubated at 37 °C for 1 h with shaking at 225 rotation per min (rpm) in an incubator shaker (Innova 4000, New Brunswick, NJ, USA). The culture was plated onto a Luria Bertani (LB) agar plate containing 50 µg/ml of kanamycin and incubated overnight at 37 °C.

Screening for recombinant transformants

Positive clones having inserts in the correct orientation were identified by colony PCR. A single colony was added into reaction mixture containing 5 µl of 10x PCR buffer, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP mix, 1.5 µl of 20 pmol SUMO forward primer (Invitrogen, Carlsbad, CA, USA) (Table 1), 1.5 µl of 20 pmol RTSV CP3 reverse primer, 0.5 µl of *Taq* DNA polymerase (5 unit/µl) (Fermentas) and sterile UHQ water to make a final volume of 50 µl. Colony PCR program used were as followed: initial denaturation at 94 °C for 8 min, 35 cycles of denaturation at 94 °C for 20 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min, final extension at 72 °C for 5 min and hold at 4 °C.

Transformation into expression host

Colonies containing insert in the right orientation were picked for culturing overnight in 8 ml of LB broth added with 50 µg/ml kanamycin at 37 °C with shaking at 225 rpm. Recombinant plasmids were isolated using PureLink Quick Plasmid Miniprep Kit (Invitrogen, Lőhne, Germany) and transformed into One Shot® BL21 (*DE3*) competent expression cells (Invitrogen, Carlsbad, CA, USA) as previously described.

Protein expression

Overnight culture of selected transformed expression *E. coli* cells was transferred into a fresh LB broth added with 50 mg/ml of kanamycin in a ratio of 1:10 and incubated at 37 °C with shaking at 225 rpm. After 2 hours, the culture was induced by adding isopropyl-b-D-thiogalactosidase (IPTG) to a final concentration of

1 mM and incubated under the same conditions for another 3 hours. Cells were harvested by cooling the cultures on ice for 5 minutes and then centrifuged in a refrigerated centrifuge (Hermle Z233MK, Germany) at 5000 x g at 4 °C for 5 min. Collected cells resuspended in 1x binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9) were disrupted by sonication (ultrasonic homogenizer 4710 Series, Cole Palmer Ins, Vernon Hills, IL, USA) to collect soluble and insoluble protein fractions for protein solubility analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Protein expressed by transformed cells with pETSUMO vector was also carried out.

Purification of recombinant RTSV CP3 protein

Based on optimised conditions for expression of recombinant RTSV CP3 protein, the recombinant protein were extracted and purified from a 100 ml culture using His-Bind column chromatography and buffers from His-Bind® Purification Kit (Novagen, San Diego, CA, USA) according to manufacturers' instructions. Concentration of purified recombinant proteins was determined by Bradford method using BioRad Protein Assay (BioRad Laboratories, CA, USA).

Analysis of recombinant RTSV CP3 protein by SDS-PAGE and Western blot

Purified recombinant RTSV CP3 protein was analysed by electrophoresis on a 12% resolving SDS-PAGE using a MiniProtean-III Cell (Bio-Rad, Hercules CA, USA). A 12% resolving gel with 4% stacking gel were prepared [15]. Protein samples to be analysed were mixed with SDS-PAGE loading buffer containing β-mercaptoethanol, boiled for 10 min and spun at 13,000 x g for 10 min. Resolved proteins on the gel were visualised by staining with Coomassie Brilliant Blue G-250 (CBB) solution or electro transferred onto a nitrocellulose membrane by in a Mini Trans-Blot module (Bio-Rad, Hercules CA, USA) [16]. The membrane was blocked with either 5% non-fat skimmed milk (NFSM) in 1x phosphate buffer saline (PBS) (5% NFSM-1x PBS) or 1% bovine serum albumin (BSA) in 1x PBS (1% BSA-1X PBS) for 30 min and subjected to three rounds of washing at 10 min interval with 1x PBS.

Due to presence of N-terminal polyhistidine (6x His)-tag in pETSUMO vector which have high affinity for nickel ions, expressed fusion proteins can be detected by HisDectector™ Nickel-horseradish peroxidase (HRP) (Ni-HRP) (KPL, MD, USA) diluted 1:500 in 1% BSA-1XPBS at room temperature for 1 h in the dark. After washing the membrane three times at 10 min intervals with 1x PBS, the membrane was developed with chromogenic substrate containing 7 mM 4-chloro-1-napthol (Sigma, St. Louis, MO, USA) in methanol/ hydrogen peroxide substrate at room temperature for 30 min in the dark. The reaction was stopped by adding distilled water. Membranes blocked with 5% NFSM-1X PBS were probed with our laboratory generated polyclonal antibodies against authentic tungro viruses from rabbit (unpublished data). Swine anti-rabbit immunoglobulins-HRP conjugate (Dako, Denmark) diluted 1:1000 in 5% NFSM-1x PBS was used as secondary probe. The membranes were developed with the same chromogenic substrate as described previously.

RESULTS AND DISCUSSION

Cloning of RTSV CP3 gene

Our study showed that template for use in amplification of RTSV CP3 gene for cloning does not need to be from a pure isolate source such as purified viruses. The gene was synthesised from total nucleic acids extracted from lysate of TN1 infected with both RTSV and RTBV. Although a few unspecific amplified fragment bands were seen, a fragment band of about 900 base pair (bp) corresponding to the PCR product size estimated at 876 bp was present as shown in **Fig.** 1.

Screening for recombinant plasmids with insert in the correct orientation by colony PCR using SUMO forward primer and RTSV CP3 reverse primer would generate a PCR product of approximately 1022 bp. The amplified PCR products would be partial SUMO protein gene and complete RTSV CP3 gene inserted. Four clones (clone 2, 8, 9 and 15) from twenty clones picked for colony PCR were screened to carry recombinant plasmid with inserts in the correct orientation (**Fig.** 2). Only a single fragment band of approximately 1 kbp was observed as shown in **Fig.** 2. Water control was clear. Thus, one in every five colonies randomly picked from LB agar plate containing 50 µg/ml of transformed Mach1™-T1® *E. coli* colonies could be carrying recombinant plasmids.

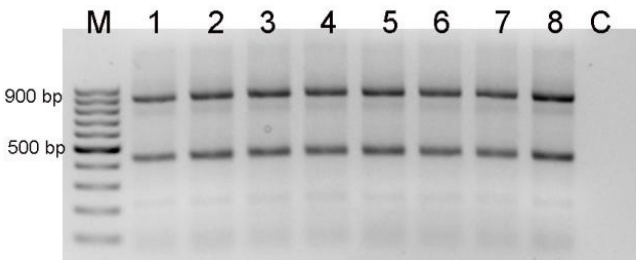

Fig. 1. PCR amplified RTSV CP3 gene. M: 100 bp DNA ladder (Fermentas) Lane 1-8: Replicate 1-8, C: Water control.

Protein expression – small scale expression and protein solubility test

Small scale expression was done to evaluate the expression level for recombinant RTSV CP3 protein. The molecular mass of RTSV CP3 protein is about 33 kDa but RTSV CP3 protein fused with 6x His-tag and SUMO protein at its N-terminal end is estimated to be approximately 46 kDa. The additional fusion protein is about 13 kDa in size. Based on CBB stained gel, recombinant RTSV CP3 protein was over expressed in the expected molecular size by three out of four positive clones screened by colony PCR (unpublished data). The three positive clones were clone 2, 8 and 15. Therefore, colony PCR result alone is not sufficient for us to conclude that a selected positive clone would express recombinant protein of interest. An additional step of either verifying the nucleotide sequence of insert or a small scale expression after the colony PCR should be performed. Presence of a stop codon at any location in the nucleotide sequence of insert could interfere with the expression result.

Clone 2, 8 and 15 were further subjected to protein solubility analysis by SDS-PAGE. As shown in **Fig.** 3, recombinant RTSV CP3 protein was over expressed in the inclusion bodies (pellet) for the three clones tested. Meanwhile, pETSUMO vector protein was expressed in the supernatant. There is a smaller fragment band of approximately 10 kDa observed for pETSUMO vector protein. This fragment band could possibly be a degraded pETSUMO vector protein. The observations were further confirmed by Western blot analysis using Ni-HRP (**Fig.** 4). Information on the solubility of the protein is important before the purification to determine the best method of purification. Protein expressed in inclusion bodies first has to be solubilised with a denaturant such as urea and then purified under denaturing conditions.

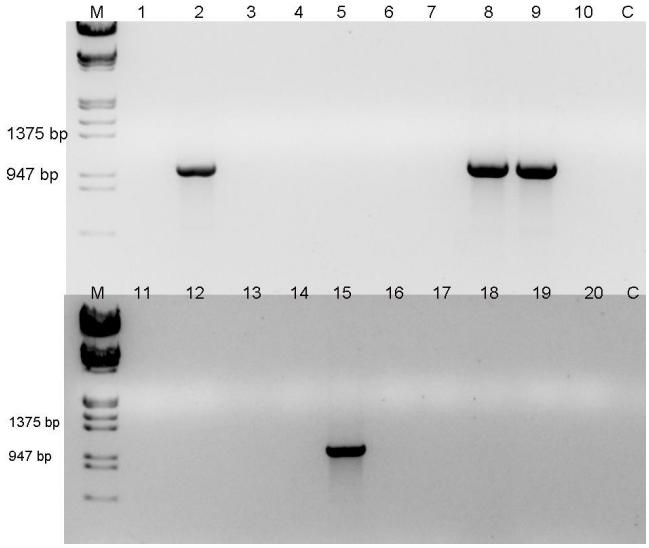

Fig. 2. Electrophorised colony PCR products. M: Lambda DNA/ *Eco*RI + *Hin*dIII marker (Fermentas), Lane 1-20: Clone 1-20, C: Water control.

Protein purification

Clone 2, being the first clone to be screened and verified, was selected for scaled-up expression. Since recombinant RTSV CP3 protein was expressed in inclusion bodies, it was purified under denaturing conditions where all the buffers used were added with 6 M urea. Purification fractions containing recombinant protein were pooled after analysed by SDS- PAGE. A final processing involving gradient dialysis (from 4 M, 2 M, 1 M, 0.5 M, 0 M urea in 0.2 M Tris-HCl, pH 8.5 and another three rounds in 1X PBS) to remove urea and to refold the recombinant protein back to its native form was carried out. pETSUMO vector protein was purified under native conditions and dialysed in 1x PBS. Total protein purified from 100 ml of culture for recombinant RTSV CP3 protein and pETSUMO vector protein are 1.872 mg and 8.713 mg respectively.

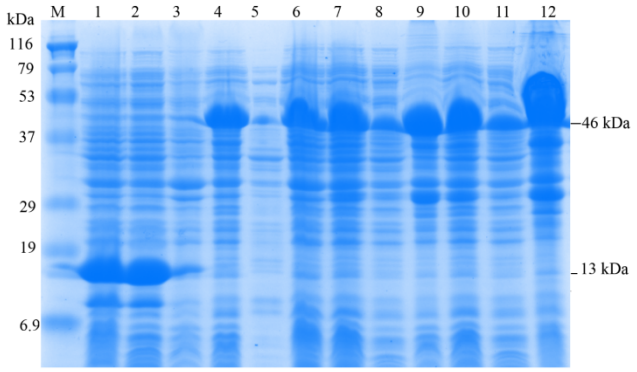

Fig. 3. Protein solubility test – CBB stained gel. Total cell protein – lane 1, 4, 7 and 10; supernatant – lane 2, 5, 8 and 11; pellet – lane 3, 6, 9 and 12; M: Pre-stained broad range marker (BioRad); Lane 1-3: pETSUMO vector control; Lane 4-6: Clone 2 - recombinant RTSV CP3 protein; Lane 7-9: Clone 8 - recombinant RTSV CP3 protein; Lane 10-12: Clone 15 - recombinant RTSV CP3 protein. All proteins were reduced and heated before electrophoresed.

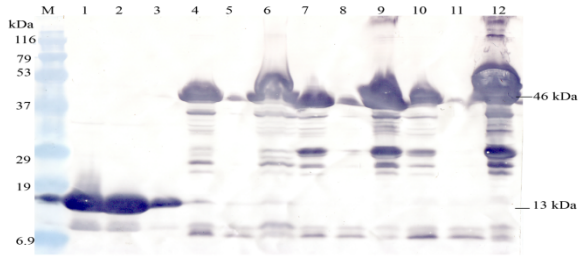

Fig. 4. Protein solubility test –Western blot membrane probed with Ni-HRP. Total cell protein – lane 1, 4, 7 and 10; supernatant – lane 2, 5, 8 and 11; pellet – lane 3, 6, 9 and 12; M: Pre-stained broad range marker (BioRad); Lane 1-3: pETSUMO vector control; Lane 4-6: Clone 2 - recombinant RTSV CP3 protein; Lane 7-9: Clone 8 - recombinant RTSV CP3 protein; Lane 10-12: Clone 15 - recombinant RTSV CP3 protein. All proteins were reduced and heated before electrophoresed.

Analysis of purified recombinant RTSV CP3 protein

Approximately 0.2 µg of purified recombinant RTSV CP3 protein was reduced with 2-mercaptoethanol and heated before electrophoresed on a 12% resolving SDS-PAGE. An equal molar mass concentration of pETSUMO vector protein was included in the analysis. CBB stained gel in **Fig.** 5(a) showed purified recombinant RTSV CP3 protein has a double band – one fragment band at approximately 46 kDa and another lower fragment band at approximately 37 kDa. No changes in the protein profile for purified pETSUMO vector protein were observed. All these were verified by Western blot analysis using Ni-HRP, and in-house rabbit polyclonal antibodies against authentic tungro viruses and negative serum as shown in **Fig.** 5 (b), (c) and (d). 6x His-tag fused to purified recombinant RTSV CP3 and peTSUMO vector proteins were detected by Ni-HRP. Our in-house rabbit polyclonal antibodies serum was able to detect the same doublet band of purified recombinant RTSV CP3 protein observed in CBB stained

gel but had no reaction with pETSUMO vector protein. Rabbit negative serum was not reactive to both proteins. The membrane probed with rabbit negative serum was clear.

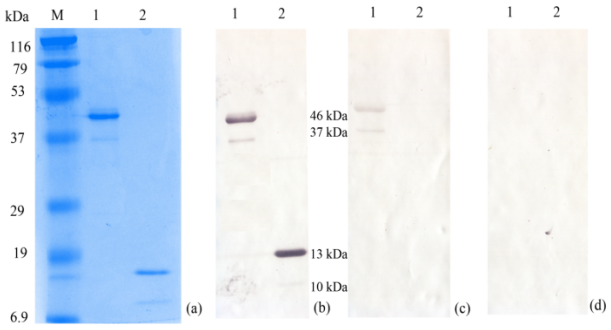

Fig. 5. Profiles of purified recombinant protein as testedby SDS-PAGE and Western blot. (a) CBB stained gel (b) Membrane probed with Ni-HRP (c) Membrane probed with in house rabbit polyclonal antibodies against authentic tungro viruses (d) Membrane probed with rabbit negative serum. M: Pre-stained broad range marker (BioRad); Lane 1: purified recombinant RTSV CP3 protein; Lane 2: purified pETSUMO vector protein.

CONCLUSION

RTSV CP3 gene was cloned successfully in pETSUMO vector with recombinant RTSV CP3 protein being over expressed in BL21 (*DE3*) *E. coli* cells. The selected purified recombinant RTSV CP3 protein was reactive with our in-house polyclonal antibodies against authentic tungro viruses. Therefore, this recombinant protein has the potential to be used as antigen in generating high titer antibodies for development of a rapid serological based diagnostic assay in detecting RTSV in a single or dual infection of RTD.

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