TECHNICAL SHEET No. 13

Virus Detection: *Potato leaf roll virus* (PLRV) CP Gene Expression

Method: RT PCR

General

Virus under study: PLRV from potato leaves.
General method for detection: RT-PCR
Gene expression strategy: Coat Protein (PLRV CP) gene based strategy.

Developed by

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Goals

Expression of the PLRV coat protein for production of ELISA kits that may use for the serological detection of PLRV in infected potato plants.

Materials and Methods

Isolation of total RNA from plant tissue

Total RNA was isolated from the infected potato plants using RNeasy® plant Mini Kit obtained from QIAGEN as the following:

1. Grind a small part of the top leaf of the infected potato to a fine powder using a sterile mortar and pestle and liquid nitrogen.
2. Transfer the tissue powder and liquid nitrogen to microfuge tube and allow the liquid nitrogen to evaporate.
3. Transfer the flow-through fraction from the QIA shredder to a new microfuge tube without disturbing the cell-debris in the collection tube, then add 225 µl ethanol (96%) to the cleared lysate and mix well by pipetting.
4. Apply the total mixture, including any precipitate, onto an Rneasy mini spin column sitting in a 2-ml collection tube and centrifuge for 15 sec at 12,000 rpm.
5. Add 700 µl of RW1 buffer onto the Rneasy column and centrifuge for 15 sec at 12,000 rpm then discard flow-through and transfer the column into a new collection tube.
6. Add 500 µl of buffer RPE containing ethanol onto the column and centrifuge for 15 sec at 12,000 rpm then discard the flow-through.
7. Add additional 500 µl of buffer RPE to the column and centrifuge at maximum speed (13,000 rpm) for 2 min to dry the column.
8. Transfer the Rneasy column into a new 1.5-ml collection tube. To elute the RNA, pipette 40 µl of RNase-free water directly onto the RNeasy membrane and centrifuge for 1 min at 12,000 rpm.

Oligonucleotide primers design and synthesis

A pair of degenerate oligodeoxynucleotide primers specific for PLRV coat protein gene were designed (M.K. Nakhla, Univ. of Wisconsin-Madison), synthesized and used for isolation, detection and gene expression of the coat protein gene. The forward primer (PLRVCPv) sequence was: 5`- AAT AGA ATT CTA ATG AGT ACG GTC GTG GTT ARA GG 3` with *Eco*RI restriction enzyme site at the 5` end, and the complementary primer (PLRVCPc) sequence was: 5`- AAA ACC ATG GCT ATY TGG GGT TYT GCA RAG CTA C – 3`, with *Nco*I restriction enzyme site at the 5` end. PCR amplification of the coat protein gene using these primers generated *Eco*R*I* and *NcoI* compatible ends (5` and 3`, respectively). The expected size of the PCR product was approximately 548 bp. The oligodeoxynucleotides were synthesized at the 50 nM scales in GIBCOBRL life technologies.

One step RT-PCR

Total RNA extracted from infected potato plants was used as templates for one-tube RT-PCR amplification reactions (one step RT-PCR system, QIAGEN). One step RT-PCR amplification was performed in a reaction volume of 50 µl.

RT-PCR mix:

10 ng/µl of total RNA
10 µl of 5x buffer
2 µl of dNTPs mix
10 µl of 5x Q-solution
3 µl of 10 µM of each primer
2 µl of QIAGEN enzyme mix
0.2 µl of Rnase inhibitor
12.8 µl DEPC treated water.

RT reaction started with incubation at 50°C for 30 min followed by denaturation at 95°C for 15 min. PCR amplification was performed by 35 cycles in thermal cycler Bio-Metra starting with denaturation at 95°C for 1 min, primer annealing at 50°C for 1 min, lastly, extension at 72°C for 1 min. and an additional extension at the end of 35^{th} cycle at 72°C for 7 min.

Electrophoresis analysis

Agarose gel electrophoresis was performed in DNA electrophoresis sub-cells (Bio-Rad). Nuseive/Seakem (3:1) molecular biology grade agarose. Agarose concentration was selected according to DNA size to be 1% and electrophoresis was performed in 1X TBE buffer. DNA samples were mixed with gel loading buffer. DNA was stained with ethidium bromide added both to the gel and the buffer at a concentration of 0.5 µg/ml. DNA was visualized on an UV transilluminator ($\lambda = 254$ nm).

Molecular cloning

The fresh PCR product was molecularly cloned in *E. coli* into pBAD TOPO-TA Expression Kit vector system from Invitrogen.

Cloning reaction:

The ligation reaction mix contained: 2 µl fresh PCR product (insert) 1 µl salt solution DEPC treated water up to 5 µl, and 1 µl vector (pBAD-TOPO). The ligation mixture was

incubated 30 min at room temperature. **Note:** The uncut control plasmid was used to check the transformation efficiency of the competent cells.

Bacterial transformation

The *E. coli* XL1-Blue competent cells were transformed by the standard method.

1. Add 5 µl of pBAD cloning reaction or 1 µl uncut control plasmid to each 50 µl competent XL1-Blue cells, and mix gently by tapping.
2. Incubate tubes on ice for 30 min then heat shock at 42ºC for 45-50 sec and place again on ice for 2 min.
3. Add 950 µl of room temperature SOC medium to each transformation mix, and incubate the tubes at 37ºC for one hour with 250 rpm shaking. From the transformation tube of the ligation mix, and 1:10 dilution of the uncut control plasmid in SOC medium, plate 100 µl on LB plates containing 50 mg/ml ampicillin.
4. Invert Plates and place them in a 37ºC incubator overnight.
5. Positive clones were identified by blue/white selection, and restriction endonucleases digestions, after plasmid purification.

Isolation of Recombinant Plasmids

Recombinant plasmids were extracted using the standard Alkaline Lysis Miniprep method.

1. Inoculate 1.5 ml of 2XYT medium containing ampicillin (60 µg/ml) with a single colony and grow overnight in shaking water bath at 37°C.
2. Centrifuge one and half milliliters of overnight culture in a 1.5-ml microcentrifuge tube at 4,000 rpm for 5 min.
3. Decant the supernatant and re-suspend the pellet in 100 µl of solution I (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM Glucose).
4. Add 200 µl of freshly prepared solution II (0.2 N NaOH, 1% SDS [sodium dodecyl sulphate] w/v) to lyse the cells
5. Add 150 µl of solution III (60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml of water) to precipitate chromosomal DNA and the large macromolecules.
6. Centrifuge the precipitated DNA at 10,000 rpm for 5 min and transfer the supernatant into a clean microfuge tube.
7. Add an equal volume of PCI to 400 µl of the supernatant and vortex for 5 min then centrifuged as in step 6, then remove the top aqueous phase to a new 1.5 ml microfuge tube
8. To precipitate the DNA add 2.5 volume (1 ml) of absolute ethanol and incubate at -80ºC for 2 h then centrifuge the precipitated DNA at maximum speed (14,000 rpm) for 15 min.
9. Wash the DNA pellet with 70% ethanol and centrifuge at 1000 rpm for 5 min then dry the pellet at the DNA vacuum concentrator.
10. Resuspend the DNA in 30 µl of TE-RNase (10 mM Tris-HCl, 1 mM EDTA, pH 8.0, 20 µg/ml RNase A) to remove about 80% to 90% of RNA from the DNA minipreparation.

Restriction digestion of plasmid DNA

Recombinant plasmids thought to contain cloned viral cDNA were digested using restriction endonuclease *Nco*I to confirm insert size.

Digestion mix:

1 µl of restriction enzyme (*Nco*I)
1 µl of 10x buffer
6 µl of DNA (mini-prep)
2 µl DEPC treated water

Mix the reaction mixture by pipetting and incubate in water bath at 37°C for 2 h. The digested DNA was electrophoresed using agarose gel (1%) and the band of interest appeared at the expected size (~580 bp).

Procedure of coat protein gene expression:

To express a gene in *E. coli* liquid LB medium was inoculated with one colony, and incubated overnight at 37 ºC to obtain a saturated culture.

1. Inoculate 50 ml of LB medium containing ampicillin with 1 ml of saturated cultures and incubate for 2 h at 37ºC.
2. Remove 1 ml of the uninduced culture and place immediately in a centrifuge tube, then spin at 12,000 rpm for 1 min at room temperature.
3. Remove the supernatant. Resuspend the pellet in 100 µl of 1x SDS gel loading buffer (50 mM TrisCl, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), and heat to 100ºC for 3 min and store at -20ºC until all of the samples have been collected and are ready to be loaded onto the PAGE.
4. Induce the remaining culture [from step 2] by adding (L)-Arabinose.
   Note: to optimize the inducer concentration; 5 different concentrations were tested in a pilot experiment. (L)-Arabinose was used at final concentrations of: 0.00002%, 0.0002%, 0.002%, 0.02%, and 0.2% [see Fig. 1].
5. Remove 1-ml aliquots of the induced culture at 2, 3, and 4 h after induction, and immediately centrifuge at 12,000 rpm for 1 min at room temperature, and discard supernatants.
6. Centrifuge the induced culture and store the bacterial pellets at -20ºC.

Protocol for purification of 6xHis-Tagged Fusion Protein Using B-PER 6xHis Spin Column Kit

1. Thaw pellets on ice before starting protein extraction. Resuspend the cell pellet in 10 ml B-PER reagent by pipetting up and down until the cell suspension is homogeneous. Once a homogenous mixture is established, shake gently at RT for 10 minutes.
2. Separate soluble from insoluble proteins by centrifugation at 14,000 rpm for 15 min.
3. Transfer the supernatant (soluble fraction) from step 2 to a 15-ml capped conical centrifuge tube. Add 1 ml the Nickel-Chelated agarose to the soluble fraction. Shake for 10 min at RT and spin at 2,500 rpm.
4. Remove supernatant and resuspend the resin with 0.25 ml wash buffer. The total volume should be about 0.75 ml.
5. Transfer the resin-bound, 6xHis fusion protein to one of the B-PER spin columns provided. Centrifuge at 10,000 rpm for 2 min.
   Note: Transfer the B-PER spin column to a new collection tube after each spin.
6. Add 0.5 ml of wash buffer to the B-PER spin column and incubate for 5 min.

7. Centrifuge at 10,000 rpm for 2 min.
8. Elute the 6xHis fusion protein by adding 0.5 ml of the elution buffer and incubating for 5 minutes. Centrifuge at 10,000 rpm for 2 min.
9. Repeat step eight 3 times for a total 4 fractions [collect each fraction into a separate collection tube]
10. The eluted 6xHis fusion protein is then assayed by SDS-PAGE [see Fig. 2].

SDS-PAGE Analysis of the expressed protein

The separating gel contains:

12.5% (w/v) acrylamide-bis acrylamide (37.5:1)
0.375 M Tris-HCl (pH 9.0)
0.1% (w/v) SDS
0.05% (w/v) ammonium persulphate
0.1% (v/v) TEMED.

The stacking gel contains:

4.0% (w/v) acrylamide-bis acrylamide
0.126 M Tris-HCl (pH 6.8)
0.1 % (w/v) SDS
0.05% (w/v) ammonium persulphate
0.1% (v/v) TEMED.

For obtaining the best results, the ammonium persulphate was prepared freshly.

1. Thaw the samples and spin down at 12,000 rpm for 1 min at RT.
2. Load 15 µl of each suspension onto 12% SDS polyacrylamide gel.
3. Stain the gel with Coomassie blue R-250 and destained using destaining solution (50% methanol and 10% acetic acid).

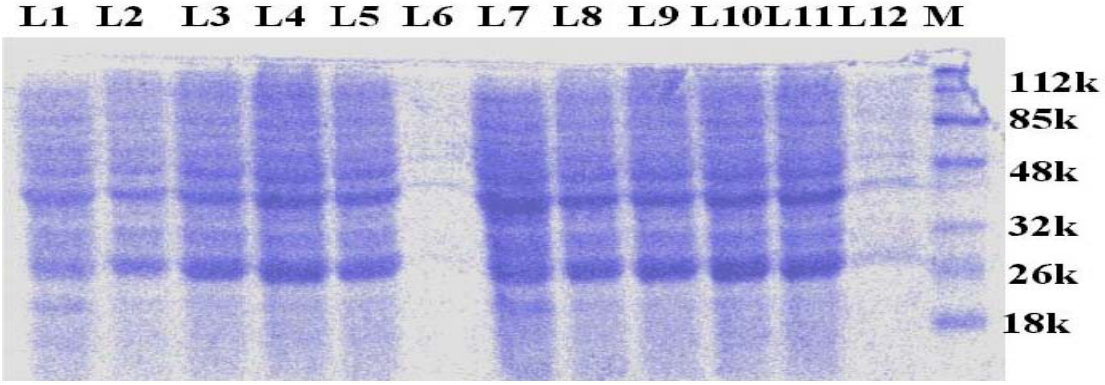

Fig 1. SDS-PAGE for induced and uninduced bacterial cells expressing PLRV CP. L1, 2, 3, 4, 5; induced cultures of the same clone at five different inducer [(L) Arabinose] concentrations; L6 is the uninduced culture of the same clone. L7, 8, 9, 10, 11 induced cultures of another clone; L12 is the uninduced culture of the same clone. The (L) Arabinose final concentrations are; 0.00002% in L5 and L11, 0.0002% in L4 and L10, 0.002% in L3 and L9, 0.02% in L2 and L8, and 0.2% in L1 and L7. M is the blue ranger prestained protein marker. Expected size was about 24KD.

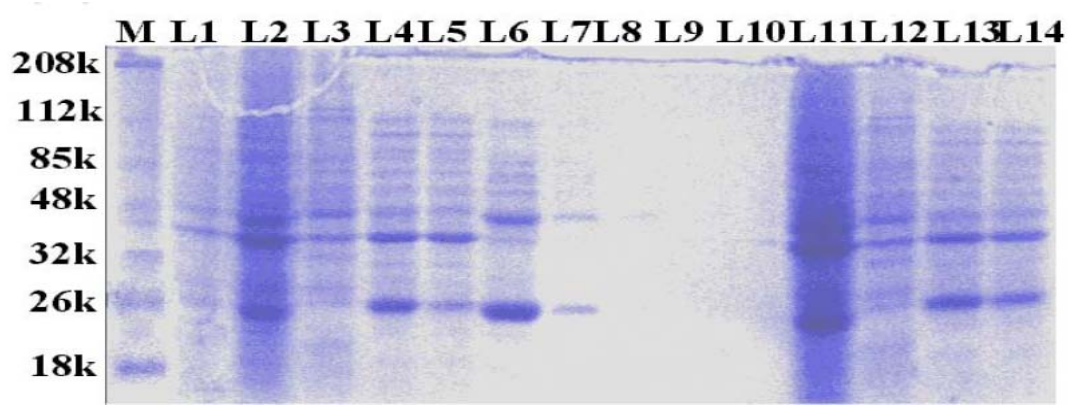

Fig 2: SDS-PAGE for induced, uninduced bacterial cells expressing PLRV CP and the purified PLRV CP. M is the blue ranger prestained protein marker, L1 uninduced bacterial culture; L2 induced bacterial culture; L3 pellet collected after chemical lysis of the induced culture (insoluble proteins); L4 Bacterial cell lysate; L5 supernatant collected after resin purification; L6 purified protein (1st elution); L7 purified protein (2nd elution); L8 purified protein (3rd elution); L9 purified protein (4th elution). Expected size of the purified protein was about 24 KD.

Conclusion

The above results demonstrated that; the best inducer concentration is 0.002% of Arabinose. The 1st elution contains the higher purified protein concentration. Elutions 3 and 4 didn't contain any proteins. Western blotting should be performed before injecting rabbits.