TECHNICAL SHEET No. 25

Virus Detection:*Plum pox virus* (PPV)

Method: RT-PCR

General

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

Developed by

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Goals

Detection of *Plum Pox Potyvirus* infected leaves and the production of polyclonal antibody specific against PPV- El Amar strain based on coat protein gene expression.

Information

Plum pox disease, Sharka, is caused by *Plum pox potyvirus* (PPV) (Dunez and Sutic, 1988; Hadidi and Candresse, 2001). Sharka is the most important of the viral diseases that affect stone fruit in Europe and the Mediterranean region because of a resultant reduced fruit quality, premature fruit drop, rapid natural spread by aphid vectors, and rapid decline and death of trees when co-infected with other viruses. During the last decade, PPV has been reported also from South and North America (Hadidi and Candresse, 2001).

Introduction of PPV through the international and national movement of stone fruit cultivars and germplasm to local stone fruit industry is of concern to federal and local governments. For this reason, effective control measures of PPV must be established to safely introduce stone fruit cultivars and/or germplasm that are free of PPV to prevent serious losses to the local stone fruit industry and significantly reduce PPV infection to stone fruits in countries where either virus is present.

Materials and Methods

Isolation of the PPV nucleic acid:

RNeasy Plant Mini Protocol (Qiagen) for Isolation of Total RNA from Plant Tissues:

1. Grind sample under liquid nitrogen to a fine powder using a mortar and pestle. Transfer to an appropriately sized tube.
2. Add 450 µl of either Buffer RLC to a maximum of 100 mg of tissue powder. Vortex vigorously. A short (1-3 min) incubation at 56ºC may help to disrupt tissue.
3. Apply lysate to the QIA shredder spin column (lilac) sitting in a 2-ml collection tube, and centrifuge for 2 min at maximum speed. Transfer flow-through fraction from QIA shredder to a new tube without disturbing the cell-debris pellet in the collection tube.

4. Add 0.5 volumes (usually 225 µ1) ethanol (96-100%) to the cleared lysate and mix well by pipetting.
5. Apply sample (usually 675 µ1), including any precipitate, which may have formed, onto an RNeasy mini spin column (pink) sitting in a 2-ml collection tube. Centrifuge for 15 sec at 10,000 rpm.
6. Pipet 700 µ1 Buffer RWI onto the RNeasy column, and centrifuge for 15 sec at 10,000 rpm to wash. Discard flow-through and collection tube.
7. Transfer RNeasy column into a new 2-ml collection tube. Pipet 500 µ1 Buffer RPE onto the RNeasy column, and centrifuge for 15 sec at 10,000 rpm. Discard flow-through and reuse the collection tube in step 8.
8. Add 500 µl Buffer RPE to the RNeasy column, and centrifuge for 2 min at maximum speed to dry the RNeasy membrane.
9. Transfer RNeasy column into a new 1.5-ml collection tube, and pipet 30-50 µl of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at 10,000 rpm to elute the RNA. Repeat if the expected RNA yield is >20µg.

RT-PCR

The RT–PCR was done by using Access RT-PCR system (Promega) with a modified PCR file according to the primer specification. The PPV coat protein primer is as follows:

PPV-CPcNcoI
ATA TCC ATG GCA TCA CAC TCC CCT CAT ACC GAG GAG G

PPV-CPvBamHI
AAY RTW GTT RTV GGA TCC GCT GAY GAR ARR GAR GAC

Electrophoretic analysis of PCR products

Following amplification, RT-PCR products were electrophoresed in agarose gels at 120V for 45 min in 0.5X TBE and visualized by staining with ethidium bromide. Product sizes were determined using the 1 kb DNA Ladder molecular weight marker (GiBCO BRL, Inc.).

Addition of 3´ A-Overhangs Post – Amplification

1. After the amplification using the RT-PCR Access System that has *Pfu* polymerase, add 1 unit of *Taq* polymerase to each tube and mix well.
2. Incubate at 72°C for 10 min.
3. Place the vials on ice. The DNA amplification product is now ready for ligation into pBAD- TOPO ® Expression vector.

TOPO Cloning Reaction

Reagent	Chemically Component *E. coli*
Fresh PCR product	3 µl
Salt Solution	1 µl
Sterile Water	1 µl
TOPO vector	1 µl

1. Mix the reaction gently and incubate at RT for 5 min.
2. Place the reaction on ice.

TOPO Transformation

1. Add 2 µl of the TOPO Cloning reaction into a vial of XL1 Blue Competent *E. coli* and mix gently.
2. Incubate on ice for 5- 30 min.
3. Heat shock the cells for 30 sec at 42°C.
4. Immediately transfer the tube to ice.
5. Add 1 ml of room temperature LB medium.
6. Shake the tube at 37 °C for 1 h.
7. Spread 50-200 µl from each transformation on a selective plate and incubate overnight at 37°C.

Mini Prep Protocol for isolation of the recombinant DNA

Mini prep was done as described in the laboratory protocol of the Geminivirus team in UW- Madison, which is a standard mini-preparation procedure for recombinant plasmids.

Evaluation of the recombinant DNA

Two methods were used for the evaluation

1. Digest using N*co*I.
2. PCR Colony with specific PCR primers for coat protein gene.

Transformation into BL21 Competent *E. coli*

The transformation was done as described above for the TOPO transformation into XL1 blue cells.

Induction Protocol - Pilot Expression Protocol

1. For each transformation inoculate 2 ml of LB containing 50 µg/ml ampicillin with a single recombinant *E. coli* colony.
2. Grow overnight at 37°C with shaking (250 rpm) to OD 600=1-2.
3. The next day, label five tubes 1 through 5 and add 10 ml LB containing 50 µg/ml ampicillin.
4. Inoculate each tube with 0.1 ml of the overnight culture.
5. Grow the cultures at 37°C with vigorous shaking (250 rpm) to an OD 600= ~0.5.
6. While the cells are growing, prepare four 10-fold serial dilutions of 20% L- arabinose with sterile water using aseptic technique (e.g. 2%, 0.2%, 0.02% and 0.002%).
7. Remove a 1 ml aliquot of cells from each tube, centrifuge at maximum speed in a microcentrifuge for 30 sec, and aspirate the supernatant.
8. Freeze the cell pellet at – 20°C. This is the zero time point sample.
9. Use the stock solution prepared in step 6 and add L-arabinose to the five 9 ml cultures

as described in the table below.

Tube	Stock Solution	Volume (ml)	Final Concentration
1	0.002%	0.09	0.00002%
2	0.02%	0.09	0.0002%
3	0.2%	0.09	0.002%
4	2%	0.09	0.02%
5	20%	0.09	0.2%

10. Grow at 37°C with shaking for 4 h.
11. Take a 1 ml sample at 4 h and treat as in step 7 and 8.

Preparing Samples for SDS-PAGE gel

1. When all the samples have been collected from Step 8 and 11, above, resuspend each pellet in 100 µl of 1X SDS-PAGE sample buffer.
2. Heat 5 min at 70°C and centrifuge briefly.
3. Load 15 µl of each sample onto an SDS-PAGE gel and separate by electrophoresis. Store the rest of the samples at -20°C.

Scale-up of Expression for Purification of coat protein

The conditions determined in the pilot expression protocol were used to grow and induce large amounts of recombinant protein.

1. Inoculate 2 ml of LB containing 50 µg/ ml ampicillin with single recombinant *E. coli* colony.
2. Grow overnight at 37°C with shaking.
3. The next day, inoculate 50 ml of LB containing 50 µg/ml ampicillin with 1 ml of the overnight culture.
4. Grow the culture at 37°C with vigorous shaking to an OD 600 = ~0.5.
5. Add the optimal amount of L-arabinose to induce expression.
6. Grow at 37°C with shaking until the optimal time point is reached. Harvest the cells.
7. Store at –80°C for future use.

Purification of 6xHis-Tagged Fusion Protein Using B-PER 6xHis Spin Column Kit

1. Prepare the bacterial lysates using either frozen or fresh bacteria from a 250 ml culture (OD 600 = 1.5-3.0). If frozen bacteria are used, thaw pellets on ice before starting protein extraction. Resuspend the cell pellet in 10 ml B-PER™ Reagent either by vortexing or pipetting up and down until the cell suspension is homogeneous. Once a homogenous mixture is established, shake gently at room temp. for 10 min. Separate soluble from insoluble proteins by centrifugation at 14,000 rpm for 15 min.
2. Transfer the supernatant (soluble fraction) from step 1 to a 15 ml capped conical centrifuge tube. Add 1ml the Nickel-Chelated agarose to the soluble fraction. Shake for 10 min at RT and spin at 2,500 rpm.
3. Remove supernatant and resuspend the resin with 0.25 ml wash buffer. The total

volume should be about 0.75 ml.

4. Transfer the resin-bound, 6xHis fusion protein to one of the B-PER™ Spin Columns provided. Centrifuge for 2 min at 10,000 rpm.
5. Add 0.5 ml of wash buffer to the B-PER™ Spin Column and incubate for 5 min. Centrifuge at 10,000 rpm for 2 min.
6. Elute the 6xHis fusion protein by adding 0.5 ml of the Elution Buffer and incubating for 5 min. Centrifuge at 10,000 rpm for 2 min.
7. Repeat 3 times for a total 4 fractions (collect each fraction into a separate collection tube)
8. The eluted 6xHis fusion protein assayed by SDS-PAGE.

Results

Fig 1. Agarose gel of RT-Polymerase Chain Reaction (PCR) amplified 900-bp fragment for *Plum pox potyvirus* (PPV) El Amar strain using primer pair (PPV-CP3 *Nco*I and PPV-CP5 *Bam*HI) to amplify the coat protein gene.

Fig 2. Agarose gel electrophoresis for *Nco*I digest of the potential recombinant plasmids from the cloned PCR fragment in Fig. 1.

Note. The *Nco*I digestions of the recombinant plasmid always give a fragment less in size than the expected one. The insert will be sequenced to provide an explanation for this *Nco*I restriction site.

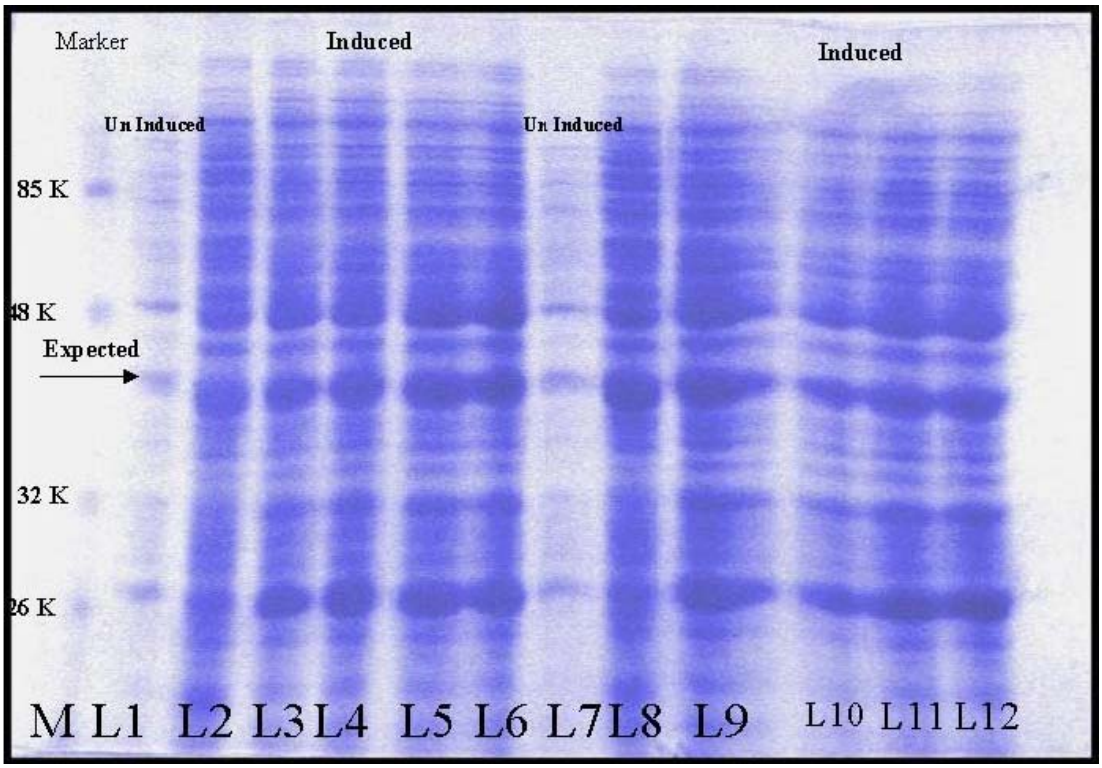

Fig 3. Marker, L1 uninduced sample, L2 induced with 20% L-Arabinose, L3 induced with 2% L-Arabinose, L4 induced with 0.2% L-Arabinose, L5 induced with 0.02% L-Arabenose,L6 induced with 0.002% L-Arabinose, L7 Uninduced, L8, 9, 10, 11, 12 induced sample using different concentrations of L- Arabinose as described before.

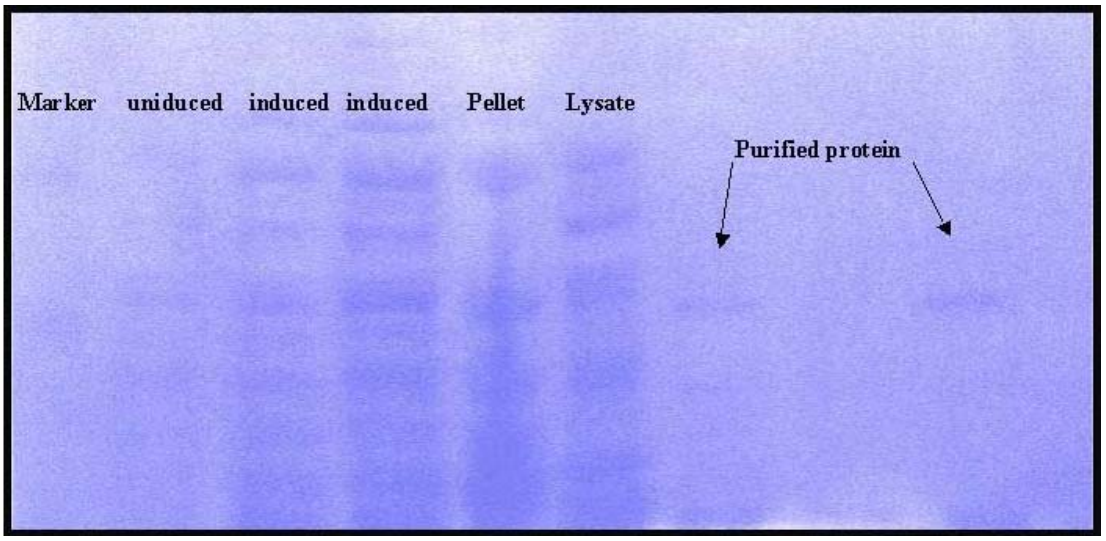

Fig 4. Polyacrylamide gel has the uninduced, induced, pellet, lysate, and purified protein in the expected size.

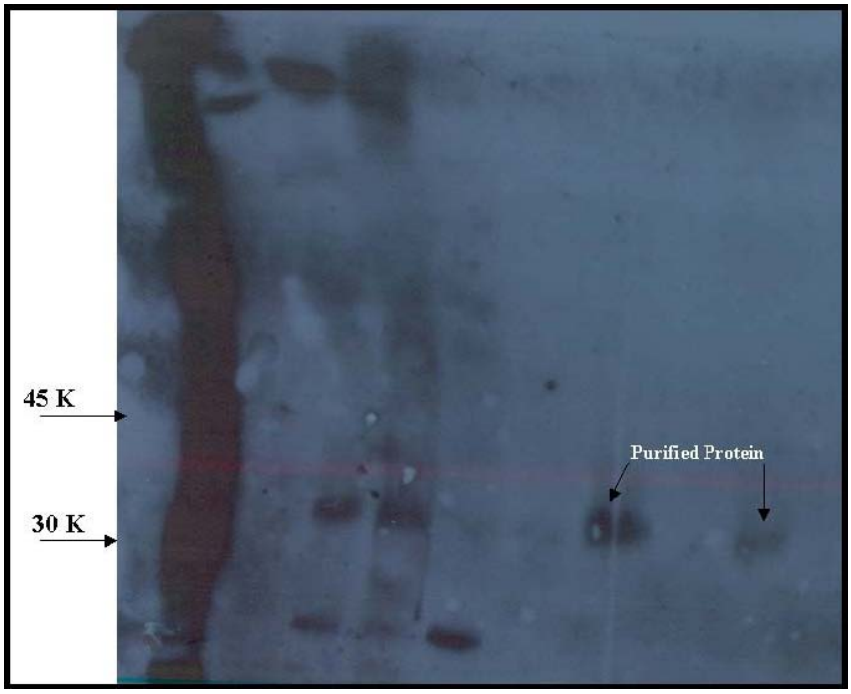

Fig 5. Western blot for the purified protein, the polyclonal antibody against PPV used to detect the expressed protein.

Discussion

The results indicated that the PPV-CP gene was isolated, cloned and expressed in *E. coli*. The expressed protein was tested by the polyacrylamide gel, and then the expressed protein was purified and tested using western blotting, which reacted positively with PPV antiserum.

References

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