Chapter 1

Production of Mosaic Turnip Crinkle Virus-Like Particles Derived by Coinfiltration of Wild-Type and Modified Forms of Virus Coat Protein in Plants

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Abstract

When the coat protein reading frame of turnip crinkle virus (TCV) is transiently expressed in leaves, virus-like particles (VLPs) are readily formed. However, after introducing genetic modifications to the full-length coat protein sequence, such as the introduction of an epitope-specific sequence within the coat protein sequence or the in-frame carboxyl terminal fusion of GFP, the formation of such modified VLPs is poor. However, by coexpression of one of these modified forms with wild-type TCV coat protein by the coinfiltration of appropriate *Agrobacterium* suspensions, VLP generation is enhanced through the formation of "mosaics," that is, individual VLPs consisting of both modified and wild-type subunits (also known as phenotypically mixed VLPs). Here we describe methods for the introduction of genetic modifications into the TCV coat protein sequence, the production of mosaic TCV VLPs and their characterization.

Key words Turnip crinkle virus, Plant infiltrations, Virus-like particles, Mosaic, *Agrobacterium*, Bionanotechnology, Transient expression, Epitope display

1 Introduction

TCV is composed of 180 copies of a single type of coat protein molecule that can adopt one of three possible structural conformations in the mature virus structure. The crystal structures of the mature particle [1] and its swollen form [2] have been elucidated. During virus assembly pairs of TCV coat protein molecules associate with each other via the coat protein Projecting (P) and Shell (S) domains (*see* Fig. 1). Coat protein pairs are then assembled into mature capsid structures by the interaction of one S domain of a coat protein pair with an adjacent S domain of another coat protein pair. The S domains of structurally related coat proteins are held together by calcium ions and it is the removal of these ions that results in the swollen form of the virus structure. In addition and as a consequence of capsid formation, each mature

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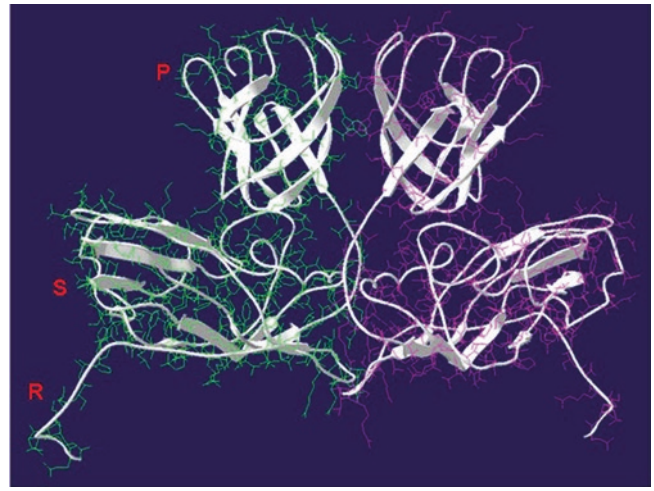

Fig. 1 The position of R (random), S (shell), and P (projecting) structural domains in an associated pair of coat protein molecules of TCV (adapted from [1])

virus particle possesses a single pair of coat protein molecules that become permanently fused together [3]. This stable dimer has been proposed to play a role in the uncoating of the mature virus particle [2].

Virus-like particles (VLPs) composed of turnip crinkle virus (TCV) coat protein can be readily generated through *Agrobacterium*-mediated transient expression using pEAQ-*HT* constructs [4, 5] containing the sequence encoding the TCV coat protein (TCVCP) [6]. Virus-sized, stable, RNA containing $T = 3$ particles result from the expression of full-length TCV coat protein; smaller, empty RNA-free $T = 1$ particles can be formed by the expression of coat protein which lacks the region that interacts with the viral genomic RNA (the random or R domain) [6]. Modified TCV VLPs that display either GFP or a hepatitis B virus epitope on the particle outer surface via the projecting (P) domain can be formed by the expression of modified forms (*see* Fig. 2) of the TCV coat protein. However, the yield of these modified VLPs is greatly diminished compared to the yield obtained with unmodified coat protein [6]. It is likely that the formation of dimers of the modified subunits is adversely affected by the additional amino acid sequences engineered into the P domain. As a result, subsequent VLP formation is not accomplished at the same rate as found with wild-type coat protein. A detailed analysis of the properties of these peptide-displaying VLPs and their potential use in bionanotechnological applications [7–9] such as vaccines [10] and as cancer targeting imaging agents [11] has been hindered as a consequence of the low yield. However, we rationalized that by performing coinfiltrations with plasmids expressing either wild-type or modified coat protein sequences it should be possible to enhance the production of peptide-displaying VLPs through the

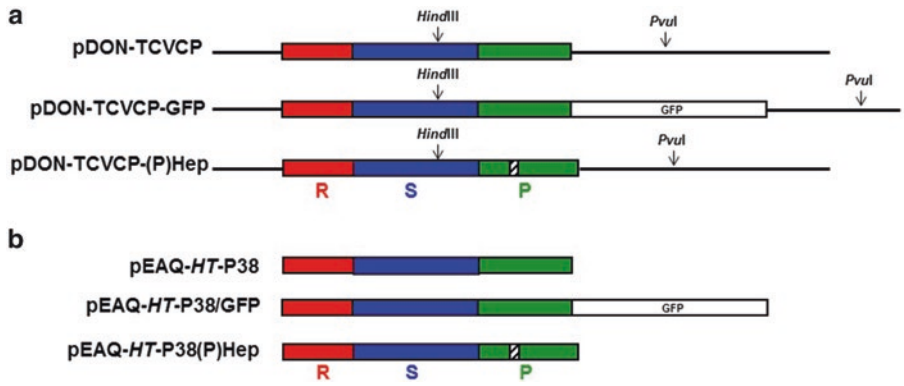

Fig. 2 Schematic representation of the gene constructions in this study. (**a**) Entry clones showing the positions of pertinent restriction sites. (**b**) Destination clones derived from the entry clones that were subsequently used to transform *Agrobacterium* LBA4404. R = random; S = shell and P = projecting structural domains, GFP = green fluorescent protein. Striped box = the location of the inserted hepatitis B epitope MIDIDPYKEFG amino acid sequence [6]

creation of mosaic particles. Using the methods described below (sucrose gradients and affinity chromatography) we have shown that the production of such mosaics, containing at least one modified subunit, significantly enhances VLP yield and is thus a route to the production of TCV VLPs displaying heterologous sequences.

2 Materials

2.1 Enzymes and Cloning Reagents

1. BP and LR clonase enzymes for Gateway cloning (Invitrogen).
2. *Hind*III, *Pvu*I restriction enzymes, Phusion polymerase, and T4 DNA ligase.
3. QIAprep spin miniprep kit (Qiagen), QIAquick gel extraction kit (Qiagen), MinElute reaction cleanup kit (Qiagen) (*see* **Note 1**).
4. Primers KS 35 and KS 36 (*see* Table 2).

2.2 Plants, Plasmids, and Bacterial Strains

1. *Nicotiana benthamiana* plants are grown in glasshouses with supplemental lighting for 16 h at a constant temperature of 24 °C. Infiltrations are performed on plants ranging from 3 to 4 weeks old (*see* **Note 2**).
2. One Shot ® TOP10 chemically competent *E. coli* (Invitrogen) is used for propagation of recombinant plasmids.
3. *Agrobacterium tumefaciens* strain LBA4404 is used for plant-based transient expression.
4. Plasmids for recombination cloning, subcloning, and *Agrobacterium* transformation are described in Table 1.
5. Bacterial glycerol stock of *A. tumefaciens* strain LBA4404 containing pBin61-TCV DNA [2, 12].

Table 1
Plasmids for TCV-like particle cloning and expression

Name	Description	Resistance
pDONR-207	Empty entry vector (Invitrogen)	Gentamycin
pDON-TCVCP	Entry vector possessing the TCV coat protein	Gentamycin
*pDON-TCVCP-GFP	Entry vector of the TCV coat protein with the GFP sequence cloned at its carboxyl terminus	Gentamycin
*pDON-TCVCP-(P)Hep	Entry vector of the TCV coat protein with the hepatitis B amino acid sequence MDIDPYKEFG cloned into the P domain of the coat protein	Gentamycin
pBin61-TCV	Infectious clone of the TCV-M strain cloned in pBin-61	Kanamycin
pEAQ-*HT*-DEST1	Empty binary vector	Kanamycin
pEAQ-*HT*-P38	Binary vector for transient TCV coat protein expression	Kanamycin
*pEAQ-*HT*-P38/GFP	Binary vector for transient TCV coat protein expression with a carboxyl terminal GFP	Kanamycin
*pEAQ-*HT*-P38(P)Hep	Binary vector for transient TCV coat protein expression with an amino acid sequence within the P domain specific for a monoclonal antibody to a hepatitis B virus amino acid sequence	Kanamycin

*Plasmids possessing modifications, addition of GFP and the insertion of the hepatitis B monoclonal antibody peptide sequence, were constructed during this study

Table 2
Oligonucleotides for cloning the TCV coat protein gene

Oligonucleotide	Sequence
KS 35	5′-GGGGACAAGTTTGTACAAAAAAGCAGG CTTAATGGAAAATGATCCTAGAGTC-3′
KS 36	5′-GGGGACCACTTTGTACAAGAAAGCTGG GTTTACTAAATTCTGAGTGCTTGC-3′

2.3 Buffers and Solutions

1. MilliQ water.
2. 2× YT medium: 16 g/l tryptone, 10 g/l yeast extract, 5 g/l, NaCl, pH 7.4.
3. SOC medium: 20 g/l tryptone, 5.0 g/l yeast extract, 0.58 g/ml NaCl, 0.19 g/l KCl, 2.03 g/l $MgCl_2$, 2.46 g/l $MgSO_4$ $7H_2O$, 3.6 g/l glucose, pH 7.5.
4. LB agar: 10 g/l Bacto tryptone, 10 g/l NaCl, 5 g/l yeast extract, pH 7.0, 10 g/l agar. Add antibiotics from stock solutions for a final concentration as indicated: LB + Kan (50 μg/ml), LB + Gen (7 μg/ml), LB + Kan (50 μg/ml) + Rif (50 μg/ml).
5. 7 mg/ml gentamycin sulfate (stock solution) in water, stored at −20 °C.
6. 50 mg/ml kanamycin sulfate (stock solution) in water, stored at −20 °C.
7. 10 mg/ml rifampicin (stock solution) in methanol, stored at −20 °C.
8. EB: 10 mM Tris–HCl, pH 8.5.
9. MMA: 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.6, 10 mM $MgCl_2$, 100 μM acetosyringone (3′, 5′-dimethoxy-4′-hydroxyacetophenone). A 100 mM stock solution of acetosyringone is prepared in ethanol and stored at −20 °C.
10. Extraction buffer: 1 mM $MgSO_4$, 1 mM sodium phosphate, pH 7.4.
11. Elution buffer: 200 μM glycine, pH 2.5, adjust pH with HCl.
12. 1 M Tris, pH 10.4, adjust pH with NaOH.
13. MOPS buffer: 50 mM MOPS, 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.7.
14. SeeBlue® Plus2 prestained protein marker (Invitrogen).
15. Instant Blue for staining NuPage gels (Expedeon).
16. Western blot transfer buffer: 25 mM Tris, 190 mM glycine, pH 8.3, 20% methanol.
17. PBS-T: 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4, pH 7.4 (adjust pH with HCl) in 1 l for a 10× stock solution, 0.05% (v/v) Tween 20.
18. 5% (w/v) skim milk powder in PBS-T.
19. Immobilon western chemiluminescent HRP substrate.
20. 25% and 70% (w/v) sucrose in 1 mM $MgSO_4$, 1 mM sodium phosphate, pH 7.4.
21. 2% (w/v) uranyl acetate.
22. NuPAGE LDS sample buffer (Invitrogen).

23. 1× TBE buffer: 10.8 g/l Tris–HCl, 5.5 g/l boric acid, 2 mM EDTA.
24. Agarose.
25. 100% (w/v) glycerol.
26. Liquid nitrogen.

2.4 Antibodies

1. Monoclonal primary antibody against the HBcAg protein epitope (ref 10E11, Abcam Ltd.).
2. Goat anti-mouse secondary antibody conjugated to horseradish peroxidase (ref W4021, Promega).
3. GFP Tag antibody conjugated to horseradish peroxidase (A10260 Invitrogen).

2.5 Consumable Materials and Devices

1. Hypodermic needles.
2. 1 ml syringes (without needle).
3. Syringe with a long needle.
4. Miracloth (Merck Chemicals Ltd.).
5. Filter paper.
6. GFP affinity chromatography columns consisting of GFP-Nano-Trap A beads (Chromotek).
7. 4–12% (w/v gradient) NuPAGE gels in NuPAGE® gel system (Invitrogen).
8. Gel transfer apparatus, wet chamber (Mini Trans-Blot®, Bio-Rad).
9. Nitrocellulose membrane (for Western blot analysis).
10. ImageQuant LAS 500 detection equipment.
11. Plastic (pyroxylin) and carbon-coated copper grids with one side flash coated with palladium (400 mesh, Agar Scientific Ltd.).
12. FEI Tecnai20 TEM microscope.
13. Eppendorf electroporator 2510.
14. Spectra-Por Float_A_Lyzer G2 dialysis devices (Spectrum Labs).

3 Methods

3.1 Genetic Modifications

For all expression studies, PCR-derived TCV cDNA from plasmid pBin61-TCV [12] was cloned into the entry vector pDONR-207 utilizing the Gateway BP recombinational ligation reaction giving rise to pDONR-TCVCP (*see* **Note 3**). This was subsequently transferred, by the Gateway LR reaction, to the pEAQ-*HT*-DEST1 expression vector [5] for plant infiltration and expression studies.

Thus, TCV VLPs are generated by expression of the full-length coat protein open reading frame from the plasmid construct pEAQ-*HT*-P38. Changes to the coat protein, by the fusion of GFP to the coat protein carboxyl terminus or the introduction of a specific sequence within the P domain of the coat protein have been accomplished with modified forms of pDONR-TCVCP. Here DNA corresponding to the necessary sequences was designed and synthesized commercially. By utilizing a unique *Hind*III site within the TCV coat protein sequence and a *Pvu*I recognition sequence flanking the coat protein sequence in the plasmid backbone, the synthetic DNA can then be ligated into pDONR-TCVCP. Modified forms of the TCV sequence in pDONR-TCVCP are subsequently transferred into pEAQ-HT-DEST1 for expression (*see* Fig. 2).

3.1.1 Cloning TCV Coat Protein Reading Frame Sequence into pDONR-207

1. Prepare plasmid pBin61-TCV DNA [2] from a −80 °C bacterial glycerol stock by inoculation of 3 ml of 2× YT medium supplemented with 50 μg/ml kanamycin. Grow at 37 °C overnight with constant agitation. Isolate plasmid DNA (Qiagen kit).
2. Set up PCR with pBin61-TCV as the DNA template, and appropriate primer pairs (KS 35 and KS 36, *see* Table 2, with reactions of 98 °C, 2 min and for 30 cycles of 98 °C for 10 s, then 65 °C for 20 s, and 72 °C for 45 s). The primer pairs are complementary to the TCV coat protein nucleotide sequence and incorporate sequences necessary for subsequent Gateway cloning (*see* Table 2). Perform PCR with Phusion polymerase.
3. Verify DNA synthesis by agarose gel electrophoresis under standard conditions on a 1.1% (w/v) agarose gel in 1× TBE buffer and recover PCR product of 1050 bp by Qiagen QIA quick gel extraction kit according to the manufacturer's instructions, eluting the DNA into EB buffer.
4. Perform the Gateway clonase BP reaction (follow manufacturer's instructions) with the PCR derived DNA and the entry vector pDONR-207. After transformation of TOP10 *E. coli*, (follow manufacturer's instructions) select for transformed *E. coli* on LB agar plates supplemented with gentamycin. Verify clones by DNA sequencing and set up the Gateway clonase LR reaction (follow manufacturer's instructions) with the destination vector pEAQ-*HT*-DEST1. Again after transformation of TOP10 *E. coli*, isolate positive transformed *E. coli* on LB agar supplemented with kanamycin. Verify positive clones by DNA sequencing.

3.1.2 Modifications to the TCV Sequence in pDONR-TCVCP

1. Design the TCV coat protein sequence to possess the GFP or other desired nucleotide sequence fused in frame to the carboxyl terminus by consulting and downloading (online) the appropriate sequence file from GenBank (accession code for

TCV: HQ589261). Assemble, in silico, such sequences to achieve the desired amino acid sequence in the expressed gene construct, *see* Fig. 2 for the location of the unique restriction sites, *Hind*III and *Pvu*I employed in this cloning procedure. Subsequently order designed synthetic sequences from a commercial synthesizer company. Similarly design the required nucleotide sequence that encodes the hepatitis B epitope sequence (MIDIDPYKEFG), or any other amino acid sequence, so that it can be inserted into the TCV coat protein P domain [6]. For both synthetic sequences, the 5′ terminal sequence and the 3′ terminal sequence will be the unique *Hind*III recognition sequence located within the TCV coat protein S domain and the unique *Pvu*I recognition sequence in the plasmid backbone respectively, Fig. 2.

2. Perform standard restriction digestions of the plasmid that contains the synthetic DNA sequence and pDONR-TCVCP with *Hind*III and *Pvu*I. Recover and purify the necessary DNAs after agarose gel electrophoresis by the Qiagen QIA quick gel extraction kit.
3. Set up ligation reactions with these DNAs with T4 DNA ligase. Transform TOP10 *E. coli*, recover and verify positive clones as before. The insert of the resulting plasmids can then be transferred into pEAQ-*HT*-Dest1 by the Gateway LR clonase reaction.

3.2 Transient Plant Expression

Agrobacterium tumefaciens is first transformed individually with the selected pEAQ-*HT*-DEST1 plasmids containing the desired nucleotide sequences, and cultures are then prepared and infiltrated into plant leaves for protein expression. Mosaic VLP generation is achieved following the coinfiltration of wild-type and the modified TCV coat protein gene constructions. By adjusting the ratio of the wild-type to the modified TCV coat protein construct in the infiltration solution, the ratio of the two forms of the protein in the mosaic VLPs can be controlled (*see* Fig. 4).

1. Store stock cultures of exponentially grown untransformed *Agrobacterium* in 25% (v/v) glycerol at −80 °C. Briefly, *Agrobacteria* cultures are collected at log phase by gentle centrifugation, washed three times, and resuspended with 100-fold concentration in 25% (v/v) glycerol. Cultures are snap frozen in liquid nitrogen prior to storage at −80 °C.
2. Transformation of *Agrobacterium* with pEAQ-based expression plasmids is achieved by electroporation [13]. Approximately 50 ng of plasmid DNA is sufficient for electroporation of 40 μl of cells at 2.5 kV. Check that a time constant between 5.8 and 6.5 μs was achieved before proceeding with the next step. If not, repeat with a fresh batch of electro competent cells. After adding 0.8 ml of SOC and incubating at 28 °C for 1 h with

shaking at 200 rpm, 10% (v/v) of the transformation mixture is spread onto LB agar containing kanamycin and rifampicin. Incubate plates at 28 °C.

3. Prepare 2× YT liquid medium with appropriate antibiotics for the *Agrobacterium* strain (rifampicin 50 μg/ml for LBA4404) and expression plasmid (kanamycin 50 μg/ml for pEAQ-based plasmids; *see* **Note 4**), to grow *Agrobacteria* for preparation of an adequate infiltration solution (*see* **Note 2**).
4. Inoculate 2× YT liquid culture, 5 ml, by picking a single colony from a plate. Grow at 28 °C in a shaking incubator until the OD at 600 nm is ≥2.
5. Centrifuge the cultures at 4000 × *g* for 10 min at room temperature to pellet the cells and discard the supernatant.
6. Resuspend the cells gently in the required volume of MMA (*see* **Note 2**) to make a solution of final OD (600 nm) = 0.4. For coexpression of two gene constructs, prepare solutions of individual OD (600 nm) = 0.8 which, when mixed 1:1, will result in a final OD (600 nm) = 0.4 for each gene construct. Ratios of 3:1 and 9:1 of wild-type TCV coat protein to modified TCV coat protein are similarly obtained by appropriate dilution of the relevant cultures (*see* **Note 5**).
7. Leave the infiltration solution at room temperature for 0.5–3 h to allow the bacterial culture to adapt to the buffer conditions.
8. Gently scratch the leaf surface with a hypodermic needle. Syringe the infiltration solution, at the damaged point on the leaf, into the leaf ensuring that the entire leaf takes up the infiltration solution (*see* **Note 6**).
9. Harvesting is typically done between 5 and 9 days post infiltration.

3.3 Extraction and Purification

1. Harvest infiltrated leaves, weigh and homogenize the leaf tissue with three volumes (e.g., for 1 g tissue, use 3 ml) of extraction buffer (*see* **Note 7**) using a blender in the cold room, at 4 °C.
2. Squeeze the homogenate through two layers of Miracloth and centrifuge at 13,000 × *g* for 20 min at 4 °C to remove cell debris.
3. To prepare the double sucrose cushion, pour the plant clarified extract into a suitable ultracentrifuge tube, then add 2 ml of 25% (w/v) sucrose solution underneath the extract by using a syringe with a long needle passing through the supernatant layer. Next add 250 μl of the 70% (w/v) sucrose underneath the previous sucrose solution by similar means. Balance the tubes and centrifuge at 274,000 × *g* for 2.5 h at 4 °C. Depending on the volume of the supernatant, use either a TH641 or Surespin rotor (or similar) [14].

4. After centrifugation, puncture the bottom of the tube with a needle (*see* **Note 8**). Recover the first 500 μl, i.e., bottom fraction (B) of the tube. Discard the next 1.5 ml and collect the next 500 μl, the middle fraction (M). Collect the supernatant fraction (S; 500 μl) directly from the top of the tube with a pipette.
5. Store samples at 4 °C for gel electrophoresis and western blot analysis (*see* Subheading 3.5).
6. For GFP affinity chromatography, ultracentrifugation fractions are dialyzed against extraction buffer overnight at 4 °C using a dialysis membrane with a molecular weight cutoff of 100 kDa. Dialyzed fractions are stored at 4 °C.

3.4 Characterization and Purification of Mosaic VLPs by Affinity Chromatography

To confirm the formation of mosaic VLPs formed of both wild-type and GFP-displaying subunits and to separate these from VLPs containing just wild-type coat protein, GFP affinity chromatography can be performed. For mosaics displaying other sequences, alternative methods specific for the inserted sequence will be required.

1. Equilibrate beads according to manufacturer's instructions (commercial product protocol). This is to ensure that the preservative in the solution in which the beads are dispatched to the customer is removed.
2. Add the dialyzed VLPs fraction (0.5–1 ml) to the equilibrated Nano-Trap®_A beads. Mix by repetitive inverting for 1 h at 4 °C on a rotating wheel.
3. Remove the bottom cap from the spin column and place it in a new 2 ml tube. Centrifuge at 100 × *g* for 10 s. Retain this fraction for subsequent gel analysis.
4. Wash the beads by resuspending them in 500 μl ice-cold dilution buffer. Place the spin column in a new 2 ml tube and centrifuge at 100 × *g* for 10 s. Collect the flow-through for immunoblot analysis and wash two more times. These fractions will consist of VLPs entirely formed of wild-type coat protein. Close the column with the bottom plug.
5. Add 100 μl of elution buffer to the nano-Trap® A beads. Pipette the beads up and down for 30 s. Remove the bottom plug of the spin column and place it in a new 2 ml tube containing 10 μl 1 M Tris pH 8.5 to neutralize the eluate. This fraction will contain mosaic VLPs (*see* **Note 9**).
6. Store samples at 4 °C for gel electrophoresis and western blot analysis (*see* Subheading 3.5).

3.5 Gel Electrophoresis and Western Blot Analysis

Protein extracts, ultracentrifugation fractions, washed and eluted chromatography fractions are analyzed by electrophoresis in 4–12% (w/v gradient) NuPAGE Bis-Tris gels resolved with MOPS buffer. In order to visualize the protein bands the gels are stained with

Instant Blue. TCV coat protein is resolved as a protein band at an apparent molecular weight of 38 kDa. Similarly, TCV coat protein fusion products are resolved at approximately 40 kDa for coat protein/Hep and at 65 kDa for coat protein/GFP respectively.

3.5.1 *Instant Blue Staining*

1. Set up 4–12% (w/v gradient) NuPAGE Bis-Tris gel according to manufacturer's instructions. Add 5 μl of SeeBlue protein marker in the first lane of the gel.
2. Gel samples, derived from the affinity chromatography procedure, are denatured by boiling for 5 min after the addition of 4× NuPAGE LDS sample buffer. Load the samples (up to 20 μl) in the remaining lanes and run the gel with MOPS buffer at 200 V for about 50 min.
3. Place the gel in Instant Blue staining solution for 1–4 h (*see* Figs. 3a and 4a). Destaining is not necessary.

3.5.2 *Western Blot Analysis*

For the western blot analysis repeat **steps 1** and **2**.

1. After electrophoresis, transfer the proteins from the gel to the nitrocellulose membrane in a gel transfer apparatus set at 100 V for 1 h using the western blot wet chamber transfer system.
2. Block the membrane overnight with 5% (w/v) milk in PBS-T with constant agitation in a cold room.

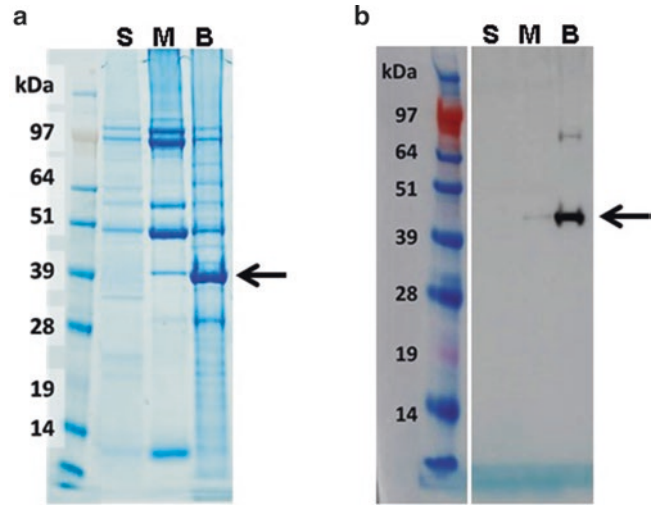

Fig. 3 NuPAGE gel separation and western blot detection of ultracentrifugation separated wild-type coat protein and wild-type/Hep fused coat proteins in mosaic VLPs. (**a**) Instant Blue staining; coat protein (molecular weight 38 kDa) arrowed. (**b**) Western blot detection of coat protein/Hep fusion product (40 kDa) arrowed. S = supernatant; M = middle and B = bottom fractions

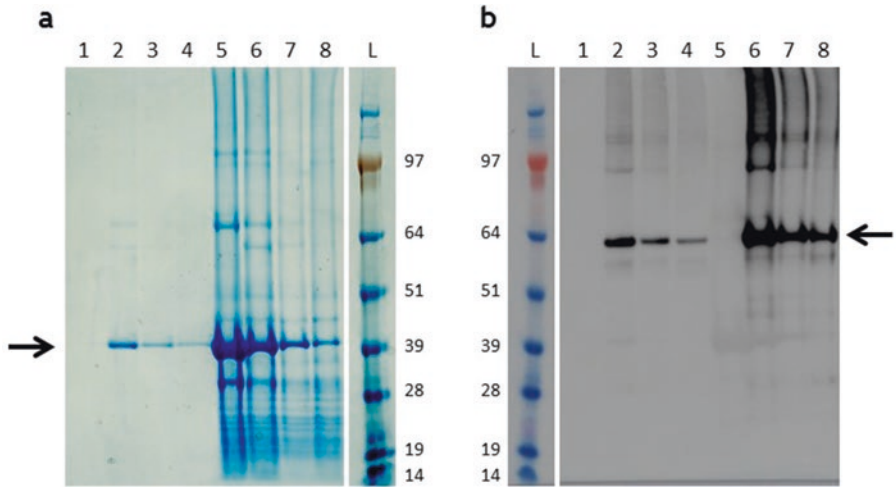

Fig. 4 NuPAGE gel separation and western blot detection of wild-type coat protein and wild-type/GFP fused coat proteins in mosaic VLPs. (**a**) Proteins revealed following NuPAGE gel electrophoresis subsequently stained with Instant Blue. Wild-type coat protein (38 kDa, arrowed) is only present in the elution fractions of the mosaic VLPs (lanes 2–4) and in plant leaf extracts (lanes 5–8) (s*ee* **Note 9**). (**b**) Western blot detection of GFP following NuPAGE gel electrophoresis. The presence of coat protein fused with GFP (65 kDa, arrowed) in the elution fractions of the mosaic VLPs (lanes 2–4) and in plant leaf extracts (lanes 6–8). Samples in lanes 1 and 5 are derived from infiltrations with pEAQ-*HT*-P38. Samples in lanes 2 and 6 are from infiltrations with a 1:1 (v:v) mixture of pEAQ-*HT*-P38 and pEAQ-*HT*/GFP. Samples in lanes 3 and 7 are from infiltrations with a 3:1 (v:v) mixture of pEAQ-*HT*-P38/GFP and pEAQ-*HT*-P38. Samples in lanes 4 and 8 are from infiltrations with a 9:1 (v:v) mixture of pEAQ-*HT*-P38/GFP and pEAQ-*HT*-P38 (*see* **Note 10**)

3. Incubate with antibody solutions diluted 1:10,000 in PBS-T is for 1 h at room temperature. Remove unbound antibody by washing three times in PBS-T for 20 min at room temperature.
   (a) To detect the hepatitis B-specific amino acid sequence, the membrane is incubated with a monoclonal primary antibody against the HBcAg protein epitope followed by its detection with a goat anti-mouse secondary antibody conjugated to HRP (*see* Fig. 3b).
   (b) GFP is detected by the use of an anti GFP-HRP conjugate antibody (*see* Fig. 4b).
4. HRP is detected with the chemiluminescent substrate ECL plus following 2 min incubation. Protein bands are subsequently visualized in an ImageQuant LAS 500 detection equipment (*see* **Note 10**).

3.6 Negative-Stained Transmission Electron Microscopy

Transmission electron microscopy can be used as a tool to confirm the assembly of virus-like particles and to study their shape and size (*see* Fig. 5).

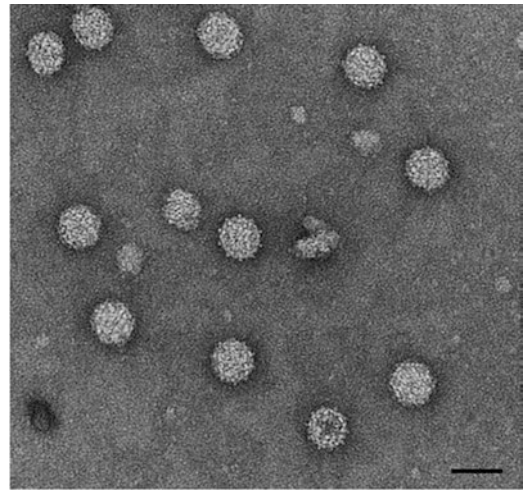

Fig. 5 TEM image of uranyl acetate stained TCV VLPs, bar = 40 nm

1. Place 5–10 μl of VLP preparation onto carbon-coated copper–palladium grids (400 mesh) and allow the particles to settle (20 s) before blotting dry with filter paper.
2. Wash the grids with five drops of MilliQ water and blot dry.
3. Negative stain the VLPs with 2% (w/v) uranyl acetate solution for 15 s then dry the grid through by blotting its edge with filter paper.
4. View grids in a transmission electron microscope such as the FEI Tecnai20 TEM microscope and obtain pictures with a bottom-mounted digital camera.

4 Notes

1. Other commercial kits for plasmid preparation, gel extraction, and enzymatic reaction cleanup can also be used.
2. The volume of the culture depends on the scale of your experiment. Generally, 5 ml of infiltration solution is enough to infiltrate three leaves of one *N. benthamiana* plant (approximately 10 g of fresh-weight tissue). If possible, prepare infiltration solution in excess to avoid being short of it during the infiltration process in the glasshouse. Typically, inoculate the culture in the afternoon and grow overnight. Resuspending the infiltration solution to an OD 600 of 0.4 usually requires 4–5 volumes of MMA to that of the starting culture.
3. For convenience, the plant expression vector pEAQ-*HT*-Dest1, a Gateway compatible vector was used throughout the current study. Restriction enzyme cloning of the TCV coat protein nucleotide sequence directly into the *Age*I and *Xho*I

restriction sites of pEAQ-*HT* [6] would have resulted in similar expression vectors.

4. Although each culture tends to grow at a different rate, allowing cultures to grow to stationary phase generally ensures that all cultures have similar densities. Other highly efficient *Agrobacterium* strains such as AGL1 may also be used, although the commonly used GV3101 (or related "nopaline" strains) are not recommended due to low-level transient expression.
5. The highest amount of fused coat protein/GFP hybrid protein was evident in preparations isolated from leaves that had been infiltrated with equal volumes of pEAQ-*HT*-P38 and pEAQ-*HT*-P38/GFP. The pH is neutralized to preserve the VLP structure.
6. The expression level is generally higher before the plant starts flowering. Plants that are 3 weeks post potting-on from the seedling stage are ideal. For small-scale experiments (useful for checking clones) smaller plants may be used for syringe infiltration of small leaf patches that can be extracted on a small scale using a bead beater or bead mill. To infiltrate leaves, nick the leaf surface with a sterile needle. Aspirate infiltration solution into a sterile 1 ml plastic syringe (take care to avoid bubbles), place the syringe over the leaf wound while keeping a finger behind the leaf for support. Gently press the solution into the intercellular space.
7. It is important not to employ buffers that contain EDTA thereby avoiding the loss of calcium from the VLPs that would result in the loss of viral structures.
8. The major concentration of VLPs is found in the interface between the 70% and 25% (w/v) sucrose solutions. Therefore, it is recommendable to take the first 0.5 ml from the bottom when using 13 ml ultracentrifuge tubes. In the middle and supernatant fractions, the presence of VLPs is low or undetectable.
9. TCV coat protein does not bind to GFP affinity beads. However TCV coat protein that has been formed into mosaic VLPs with TCV coat protein/GFP will be eluted from GFP affinity beads due to the interaction between TCV coat protein fused to GFP and the beads. Therefore in lane 1 Fig. 4a, there is no TCV coat protein observed because these VLPs are entirely formed of wild-type coat protein.
10. We have demonstrated the formation of mosaic VLPs through the coexpression of both wild-type and modified coat proteins. This approach has clear benefits, compared to infiltrations with just the modified coat protein [6]. The formation of mosaic VLPs by other plant and animal viruses, whose capsid is formed of repeated single coat protein molecules, like TCV, is worthy of investigation.

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