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ORIGINAL ARTICLE

Assembly of tomato blistering mosaic virus-like particles using a baculovirus expression vector system

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Abstract

The expression of several structural proteins from a wide variety of viruses in heterologous cell culture systems results in the formation of virus-like particles (VLPs). These VLPs structurally resemble the wild-type virus particles and have been used to study viral assembly process and as antigens for diagnosis and/or vaccine development. Tomato blistering mosaic virus (ToBMV) is a tymovirus that has a 6.3-kb positive-sense ssRNA genome. We have employed the baculovirus expression vector system (BEVS) for the production of tymovirus-like particles (tVLPs) in insect cells. Two recombinant baculoviruses containing the ToBMV wild-type coat protein (CP) gene or a modified short amino-terminal deletion ($\Delta_{2\text{-}24}$CP) variant were constructed and used to infect insect cells. Both recombinant viruses were able to express ToBMV CP and $\Delta_{2\text{-}24}$CP from infected insect cells that self-assembled into tVLPs. Therefore, the N-terminal residues (2-24) of the native ToBMV CP were shown not to be essential for self-assembly of tVLPs. We also constructed a third recombinant baculovirus containing a small sequence coding for the major epitope of the chikungunya virus (CHIKV) envelope protein 2 (E2) replacing the native CP N-terminal 2-24 amino acids. This recombinant virus also produced tVLPs. In summary, ToBMV VLPs can be produced in a baculovirus/insect cell heterologous expression system, and the N-terminal residues 2-24 of the CP are not essential for this assembly, allowing its potential use as a protein carrier that facilitates antigen purification and might be used for diagnosis.

Introduction

Tomato blistering mosaic virus (ToBMV) is a member of the genus *Tymovirus* and the family *Tymoviridae* and contains a monopartite positive-sense ssRNA genome [1]. ToBMV causes a severe disease worldwide in plants of the genus *Solanum*, with symptoms that include leaf mosaic and blistering [2]. Tymovirus virions are isometric, non-enveloped, and approximately 30 nm in diameter, arranged in a T = 3 icosahedron structure with clustering of coat protein subunits in pentamers and hexamers protruding from the underlying surface [3]. A subgenomic promoter composed of a 16 conserved nucleotides, referred to as the "tymobox", is responsible for the expression of CP [4]. Viral replication takes place in the cytoplasm in association with the double membrane-bounded vesicles that line the periphery

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of the chloroplasts and/or mitochondria [3, 5]. Interestingly, previous studies have shown that the CP of physalis mottle virus (PhyMV) and turnip yellow mosaic virus (TYMV) are able to self-assemble into virus-like particles (VLPs) when expressed in *Escherichia coli* [6, 7].

VLPs can be obtained by the expression of viral structural proteins in several bacterial and eukaryotic heterologous systems [8]. They are almost identical in structure and morphology to virus particles but lack a genome and are therefore noninfectious [9]. VLPs are a highly immunogenic and safe alternative to inactivated infectious viruses. They have already been used as vaccines against several viruses, such as human papilloma virus (HPV) [10], hepatitis B virus (HBV) [11], and influenza A virus [12]. Importantly, the baculovirus expression vector system (BEVS) is a well-established eukaryotic-expression-system-based technology that is chosen for the production of a large number of recombinant proteins and is becoming one of the most powerful, robust, and economical systems for protein expression [13]. The BEVS can be used for the production of VLPs, including those from plant viruses [14]. However, despite being a versatile and powerful tool to express different foreign proteins, it is challenging to recover and purify the recombinant protein [15]. To overcome this problem, several strategies focus on tagging the recombinant proteins with carrier proteins or peptides to facilitate protein purification [16].

In this work, we constructed three recombinant baculoviruses to express different variants of the ToBMV CP in insect cells, with the aim of producing ToBMV VLPs (tVLPs). To our knowledge, this is the first study that evaluates tymovirus-like particle assembly using a BEVS and its potential as a carrier structure for short epitopes to facilitate production and purification.

Materials and methods

Construction of recombinant bacmids containing the ToBMV *cp* gene and its variants

Three different CP variants were generated to construct the recombinant baculoviruses: (i) the complete *cp* of ToBMV (CP variant); (ii) a mutant of the ToBMV *cp* (23-amino-acid deletion in the amino-terminal region of the predicted CP protein) ($\Delta_{2\text{-}24}$CP variant); and (iii) a mutant fusion with the E2 epitope of chikungunya virus (CHIKV) (QRRSTKDNFNVYKATRPYLAHCPDC; E2-ΔCP variant). As a DNA template for the PCR reaction, we used a previously constructed cDNA clone, pToBMV (accession number KT834406) [17]. For CP amplification, we used the primers ToBMV-CP-F (5'-AAG CTT GAA TTC ATG GAT CCC AGT ACT TCA AAC ACC ATC-3') and ToBMV-CP-R (5'-CCA TGG CTG CAG CTA GGA GGA TTG AAG AAG AGG AGA GGA-3'). For $\Delta_{2\text{-}24}$CP, we used ToBMV-CP-Del-F (5'-AAG CTT GAA TTC ATG ACC CCC ACC ACT GAA CTC TCC GGA GC-3') together with ToBMV-CP-R. For E2-$\Delta_{2\text{-}24}$CP amplification, we performed a two-step PCR for subsequent addition of the E2-epitope-encoding nucleotide sequence to the $\Delta_{2\text{-}24}$CP variant. In the first step, we used the primer F2 (5'-TTT ACA AGG CCA CTA GAC CAT ACT TGG CCC ACT GCC CAG ATT GTA CCC CCA CCA CTG AAC TCT CCG GAG CCA TTG-3') together with ToBMV-CP-R and, as a template, pToBMV. F2 binds to the $\Delta_{2\text{-}24}$CP variant of the CP gene and inserts a partial E2 epitope sequence. The resulting fragment was used as a template in a second reaction with the primer F2 (5'-AAG CTT GAA TTC ATG CAA CGT CGT TCA ACA AAA GAC AAT TTT AAT GTT TAC AAG GCC ACT AGA CCA TAC TTG GCC-3') together with ToBMV-CP-R. The purified fragments were individually digested with *Eco*RI/*Pst*I and separately cloned into pFastBac™1 (Invitrogen, Carlsbad, CA, USA) digested with the same enzymes to generate three transfer vectors. The transfer vectors were individually transferred into DH10-Bac cells (Invitrogen, Carlsbad, CA, USA) by heat shock. Recombinant bacmids were selected and confirmed by PCR following the manufacturer's instructions (Bac-to-Bac®, Baculovirus Expression Systems, Invitrogen, Carlsbad, CA, USA). One µg of each recombinant bacmid was induced by transfection into *Trichoplusia ni* (BTI-Tn5B1-4, herein called Tn5B) cells (10^6) [18] using liposomes (Cellfectin®) according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Tn5B cells were cultured at 27°C in TC-100 medium (Vitrocell, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (Thermo Fisher, Walthan, MA, USA). The supernatant of cells containing the recombinant viruses vAc-ToBMV-CP, vAc-ToBMV-$\Delta_{2\text{-}24}$CP and vAc-ToBMV-E2-$\Delta_{2\text{-}24}$CP were collected at seven days post-transfection, amplified in Tn5B cells, and titered as previously described elsewhere [19].

Protein expression analysis in Tn5B cells

For the infection of Tn5B cells with baculovirus, the viral stock was added to the cells (1-2 × 10^6 cells/mL) at a multiplicity of infection (MOI) of 5. At four days postinfection (d.p.i.), the infected cells and the culture supernatant were separated by centrifugation at 5,000 × *g* for 5 min. The cells were washed with phosphate-buffered saline (PBS, 3 mL; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4; pH 7.4) and pelleted by centrifugation at 5,000 × *g* for 5 min. Western and dot blot analyses were used to evaluate the specificity of ToBMV CP variants using a ToBMV CP-specific polyclonal antibody (kindly provided by Alice K. Inoue-Nagata, Embrapa Hortaliças, Brasília, Brazil). For Western blot, the pellet was resuspended in PBS, and the total protein was quantified by the Bradford method

(Bio-Rad, Hercules, CA, USA) according to the manufacture's protocol. Cell extract samples were mixed with equal volumes of 2x protein loading buffer (0.25 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.02% bromophenol blue) and incubated at 100 °C for 5 min. Samples were resolved by 12% SDS-PAGE [20], transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Burlington, MA, USA), and probed with a rabbit anti-ToBMV-CP polyclonal antibody. The probing was followed by incubation with alkaline-phosphatase-conjugated anti-rabbit secondary antibody (Sigma-Aldrich, St. Louis, MO, USA). Blots were developed using alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$; pH 9.5) containing 3.5 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma-Aldrich, St. Louis, MO, USA) and 3.7 mM nitro blue tetrazolium (NBT) (Sigma-Aldrich, St. Louis, MO, USA). Leaf extracts of ToBMV-infected symptomatic *Nicotiana benthamiana* were used as positive control, while control virus vAc-occ- (occlusion-negative Autographa californica multiple nucleopolyhedrovirus) [16] and uninfected Tn5B cells were used as a negative control. For dot blot analysis, the crude cell extracts, lysate and purified proteins were manually dotted on a nitrocellulose membrane and probed using the same procedure as above.

Light and immunofluorescence microscopy

In order to examine the cytolocalization of the expressed CP variants in insect cells during baculovirus infection, we performed an immunofluorescence (IF) assay using infected Tn5B cells and anti-ToBMV-CP antibody. Monolayers of Tn5B (5.0×10^6) cells were infected at an MOI of 5 with recombinant vAc-ToBMV-CP, vAc-ToBMV-$\Delta_{2\text{-}24}$CP, or vAc-ToBMV-E2-$\Delta_{2\text{-}24}$CP, and after 72 h p.i., the cells were fixed with 3.7% formalin and incubated for 1 h with a 1:200 dilution of polyclonal rabbit anti-ToBMV-CP in PBS. After the washing, the cells were incubated with fluorescent Alexa Fluor 488 anti-rabbit IgG secondary antibody (Thermo Fisher, Walthan, MA, USA). Images were taken with a Zeiss Apotome Axiovert 200 (Zeiss, Munich, Germany) and processed with AxioVision 4.8.

Purification, negative staining, and transmission electron microscopy of tVLPs

Infected cells were harvested from an 80% confluent 72-h-old culture in a T75 culture flask and centrifuged at 5,000 × *g* for 5 min. The resulting pellets containing the infected cells were washed twice with PBS, followed each time by centrifugation at 5,000 × *g* for 5 min. The cells were then resuspended in 500 µL of PBS and lysed by sonication on ice for 15 s (Sonics Vibracell, output 2 W). The lysate was clarified by centrifugation at 5,000 × *g* for 5 min., and the supernatant was subjected to ultracentrifugation for 3 h at 100,000 × *g* onto a sucrose cushion (25% sucrose in PBS). The pellets were resuspended in 500 µL of PBS, and a small drop of each sample was spotted onto a carbon-coated copper grid and negatively stained with 2% (w/v) uranyl acetate. The grids were examined using a transmission electron microscope (JEOL JEM 1011, JEOL, MA, USA).

Results

Virus construction and protein expression

Two transfer vectors containing the *cp* variants were used to construct the recombinant baculoviruses vAc-ToBMV-CP and vAc-ToBMV-$\Delta_{2\text{-}24}$CP. We observed baculovirus cytopathic effects when Tn5B cells were infected with either vAc-ToBMV-CP or vAc-ToBMV-$\Delta_{2\text{-}24}$-CP that were similar to those observed with the control virus, vAc/ooc-. The cytopathic effects included hypertrophy of the nucleus and cell rounding (Fig. 1A). The infected cells were harvested and tested for protein expression by immunoblot analysis (Fig. 1B). The proteins were detected using an anti-ToBMV-CP antibody (anti-CP). The predicted molecular mass of the CP protein (19.8 kDa) and $\Delta_{2\text{-}24}$-CP (17.4 kDa) corresponded to the immunoreactive band observed in the immunoblot. No bands were detected in uninfected or vAc/ooc-infected cell extracts.

Immunofluorescence analysis of infected cells

Uninfected Tn5B cells exhibited very low background fluorescence, while infected cells expressing the CP variants were labeled (Fig. 2). IF analysis showed that both CP and $\Delta_{2\text{-}24}$-CP variants accumulated in the cytosol during virus infection.

Tymovirus-like particle (tVLP) formation during baculovirus infection

We used dot blot analysis to probe both the crude extract and the pellet obtained by ultracentrifugation with a ToBMV-CP antibody (Fig. 3A). Reactive dots were found in crude extract and purified putative tVLPs from the two variants. We found no signals with the extract from uninfected cells. For analysis of tVLP assembly, we performed negative staining [21] on the pellet material and examined the putative VLPs using transmission electron microscopy (Fig. 3B, black arrowhead). The sizes of the VLPs were not regular and varied from 10 to 50 nm. Both variants, CP and $\Delta_{2\text{-}24}$-CP, formed icosahedral virus-like particles that resembled tymovirus capsids (the native virus is not shown), confirming that the recombinant proteins expressed in

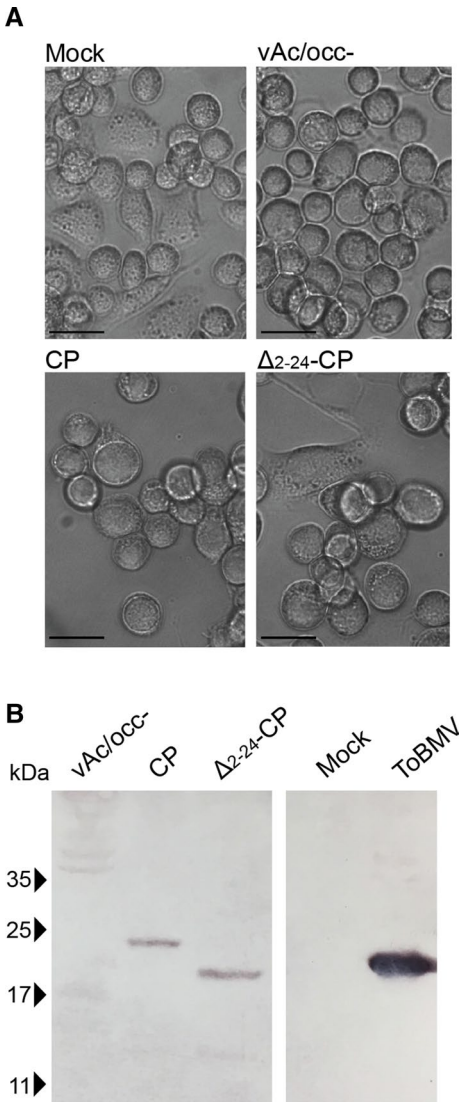

Fig. 1 Expression of recombinant tomato blistering mosaic virus (ToBMV) coat protein (CP) analyses. (**A**) Uninfected Tn5B cells and Tn5B cells infected for 72 h with vAc/occ-, vAc-ToBMV-CP, or vAc-ToBMV-$\Delta_{2\text{-}24}$-CP. The infected cells display hypertrophy of the nucleus, cell rounding, and loss of the fusiform shape observed in uninfected cells. Scale bar = 50 µm. (**B**) Western blot showing the presence of specific bands corresponding to the recombinant CP variants expressed during virus infection

baculovirus were capable of assembling into VLPs (Fig. 3B, black arrowhead). Negative staining revealed rod-shaped baculovirus capsids (Fig. 3B, CP field, white arrowhead).

ToBMV cp carrying the CHIKV epitope

To determine whether tVLPs are capable of carrying small peptides, we evaluated the assembly of tVLPs containing an immunogenic E2 epitope of CHIKV fused to the $\Delta_{2\text{-}24}$-CP variant of the ToBMV CP. The transfer vector sequence was confirmed by DNA sequencing and used to construct a recombinant baculovirus, vAc-ToBMV-E2-$\Delta_{2\text{-}24}$-CP. Typical cytopathic effects of baculovirus infection were also observed in Tn5B cells after infection (Fig. 4A). The recombinant protein E2-$\Delta_{2\text{-}24}$-CP was detected using an anti-ToBMV-CP antibody (anti-CP) (Fig. 4B). The predicted molecular mass of the E2-$\Delta_{2\text{-}24}$-CP with an additional 25 amino acid residues at its N-terminus (20.3 kDa) is similar to that of the immunoreactive band observed in the Western blots (Fig. 4B). No band was detected in either vAc/occ- and uninfected cell extracts (not shown). We also performed an IF analysis of vAc-ToBMV-E2-$\Delta_{2\text{-}24}$-CP-infected Tn5B cells at 72 h p.i. We found that the E2-$\Delta_{2\text{-}24}$-CP protein of ToBMV accumulated in the cytosol of Tn5B cells, as observed for CP and $\Delta_{2\text{-}24}$-CP (Fig. 4C). The results of dot blot analysis showed that the ToBMV CP antibody reacted strongly with crude lysates, and purified extracts of cells infected with vAc-ToBMV-E2-$\Delta_{2\text{-}24}$-CP after ultracentrifugation of the infected cell lysate onto a sucrose cushion (Fig. 4D). Importantly, TEM examination of purified extracts of Tn5B cells infected with vAc-ToBMV-E2-$\Delta_{2\text{-}24}$-CP revealed icosahedral virus-like particles resembling typical tymovirus virions, showing that the recombinant protein E2-$\Delta_{2\text{-}24}$-CP carrying a heterologous small peptide sequence was able of self-assembly into a VLP (Fig. 4E).

Discussion and conclusions

The last two decades have witnessed the growing use of the eukaryotic baculovirus expression system to produce a great number of recombinant proteins [22]. In this study, we expressed the ToBMV coat protein (CP) using this system for the production of tymovirus-like particles (tVLPs). The expression system enabled the formation of tVLPs from two different CP variants: a full-length variant (CP) and an amino-terminal deletion mutant lacking 23 amino acids ($\Delta_{2\text{-}24}$-CP) of the native CP. We also found that the fusion of a small peptide based on the envelope protein E2 of the CHIKV did not change the self-assembly properties of a deletion mutant of ToBMV CP.

The use of plant virus-like particles as carrier proteins is a very promising approach for displaying epitopes and facilitating their purification, taking advantage of the high density of the particles [23]. Members of different families of plant viruses have been used to produce VLPs using the baculovirus expression vector system (BEVS), including arabis mosaic virus [24], tobacco ringspot virus [25], cowpea mosaic virus (CPMV) [26], beet western yellows virus [27], potato leaf roll virus [14, 28], pea enation mosaic virus [29], and rice dwarf virus [30]. None of those viruses are members of the genus *Tymovirus* or were used as a carrier structure to carry epitopes. The expression of tobacco ringspot virus (TRSV) coat protein in insect cells using a BEVS enabled the formation of empty VLPs that were similar in

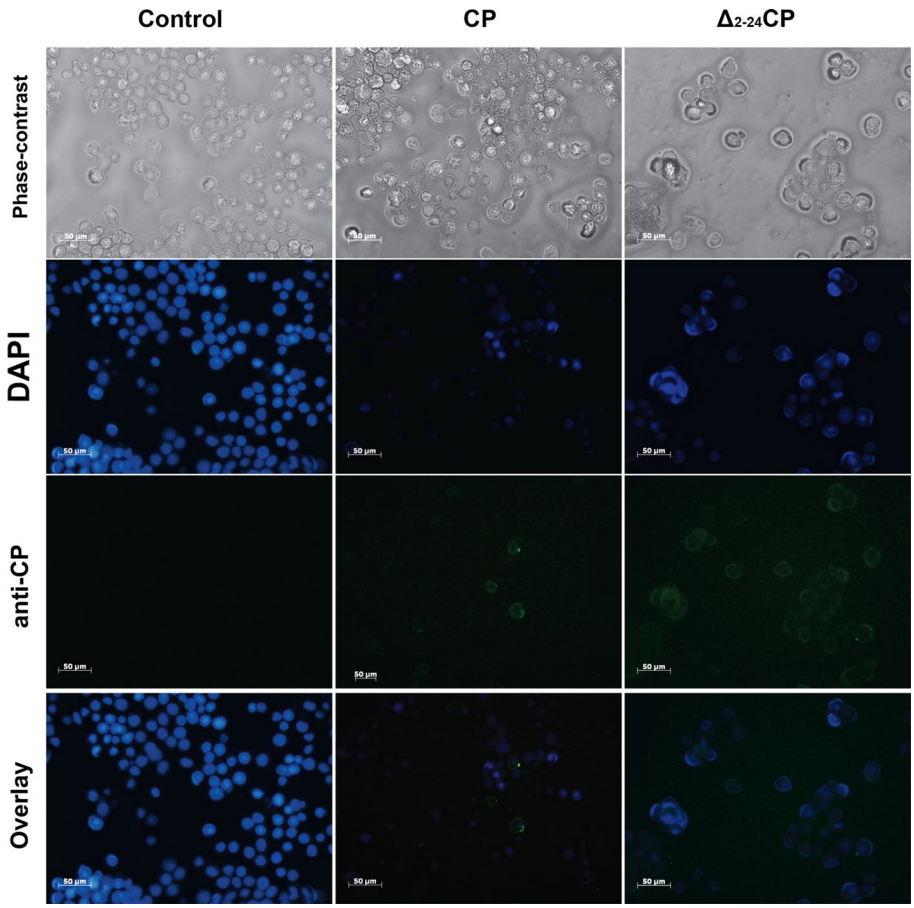

Fig. 2 Immunofluorescence analysis of Tn5B cells infected with recombinant baculoviruses expressing tomato blistering mosaic virus coat protein (ToBMV CP) variants. The figure shows Tn5B cells infected with either vAc-ToBMV-CP or vAc-ToBMV-$\Delta_{2\text{-}24}$-CP at 72 h p.i. and incubated with rabbit polyclonal antibodies raised against the ToBMV CP protein, after DAPI staining to show nuclear localization

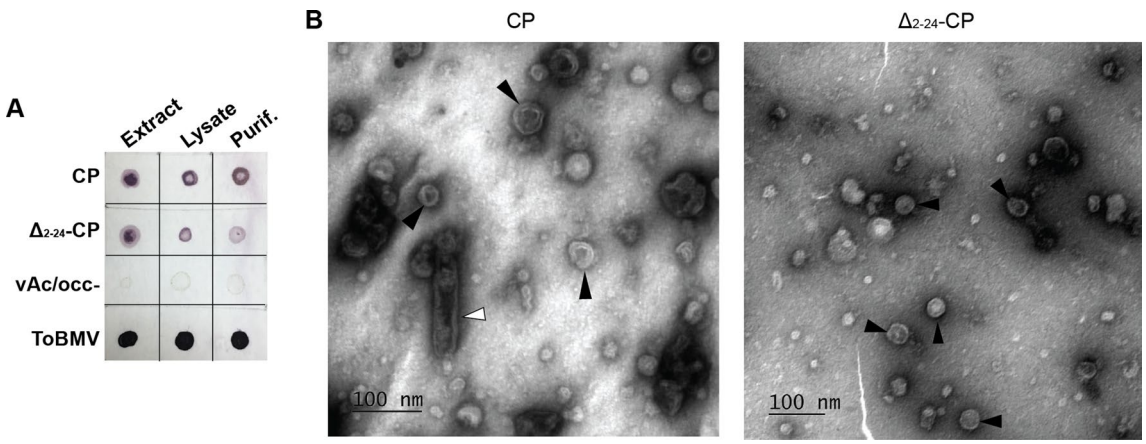

Fig. 3 (**A**) Dot blot analysis of the crude lysates and purified extracts from infected cells using vAc-ToBMV-CP or vAc-ToBMV-Δ2-24-CP recombinant viruses and anti-ToBMV-CP antibody. Visible positive signals are present in the lanes containing CP, but not in the vAc-occ- (negative control) lane, for the total extract, the lysate, and the purified particles. (**B**) Electron micrographs of virus-like particles (VLPs) assembled during vAc-ToBMV-CP or vAc-ToBMV-$\Delta_{2\text{-}24}$-CP infection. The tVLPs are indicated by a black arrowhead. White arrowheads indicate rod-shaped baculovirus nucleocapsids. Bars in panels **B** and **C**, 100 nm

structure to the native virus particles [25]. Interestingly, in the case of CPMV, two coat proteins were expressed either individually or together in insect cells using a BEVS. Only co-expression from separate promoters in the same construct resulted in the formation of VLPs whose morphology closely resembled that of native CPMV virions [26]. Furthermore, the coat protein of beet western yellows virus (BWYV) was expressed using recombinant Bombyx mori

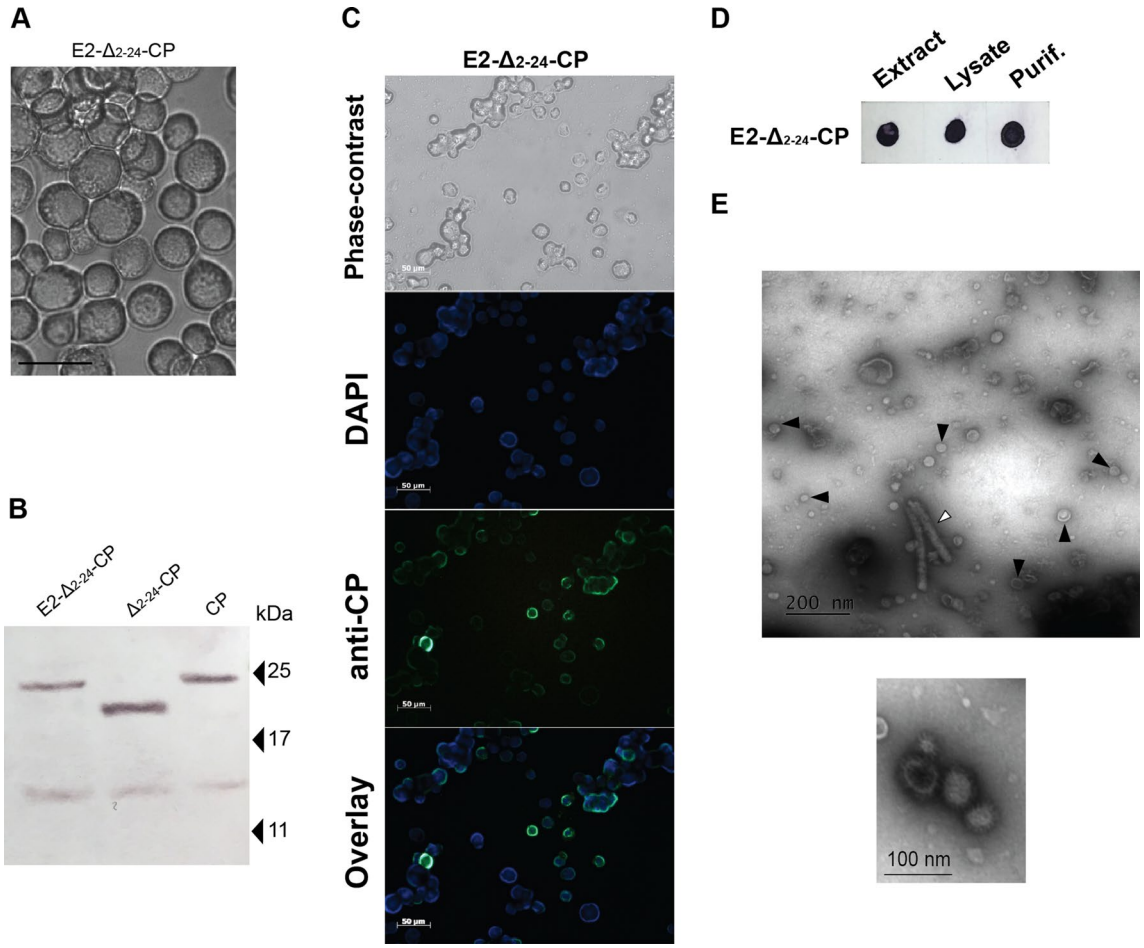

Fig. 4 Analysis of recombinant E2-$\Delta_{2\text{-}24}$-CP protein expression. (**A**) Cytopathic effects induced by the infection of recombinant Tn5B cells with E2-$\Delta_{2\text{-}24}$-CP. (**B**) Western blot showing the presence of specific bands corresponding to CP variants expressed during recombinant baculovirus infection. (**C**) Immunofluorescence analysis of Tn5B cells infected with vAc-ToBMV-E2-$\Delta_{2\text{-}24}$-CP baculovirus. The figure shows Tn5B cells infected at 72 h p.i. and incubated with rabbit polyclonal antibodies raised against the ToBMV CP protein and DAPI staining to show nuclear localization. (**D**) Dot blot analysis of crude lysates and purified extracts from infected cells, using vAc-ToBMV-E2-$\Delta_{2\text{-}24}$-CP recombinant virus and anti-ToBMV-CP antibody. Visible positive signals are present in the three dots. The negative control is not shown, as it did not generate any signal. (**E**) Electron micrographs of virus-like particles (VLPs) assembled from vAc-ToBMV-E2-$\Delta_{2\text{-}24}$-CP infection. The tVLPs are indicated by a black arrowhead, which is highlighted in the additional box below. White arrowheads indicate rod-shaped baculovirus nucleocapsids

nucleopolyhedrovirus (BmNPV) *in vivo*. Fat body cells from *Bombyx mori* larvae infected with recombinant BmNPV produced particles resembling BWYV virions in their cell nuclei [27].

The ability of tymovirus CP to self-assemble in heterologous expressions system was previously assessed for PhyMV and TYMV CPs. The tVLPs were able to assemble upon CP expression in *E. coli*, even with N-terminal deletions in the protein [6, 7, 31, 32]. Importantly, the flexible N-terminus of the CP of both PhyMV and TYMV was shown not to be essential for tVLP formation [33, 34]. In previous work, fusion of immunodominant epitopes of the Japanese encephalitis virus envelope protein to the CP of PhyMV resulted in efficient expression in *E. coli* and assembly into VLPs with a very similar structure to those found in this work [34]. The tVLPs expressed in BEVS varied in size from 10 to 30 nm. When the pea enation mosaic virus (PEMV) CP was expressed in insect cells from a recombinant baculovirus, VLPs were produced that varied in size from 13 to 30 nm.

The BEVS is highly versatile platform for the production of structurally and immunologically functional VLPs [22] and protein display. There are many strategies using the budded virus as a fusion partner or for surface presentation of target proteins in insect cells (the major envelope protein and the capsid protein). Furthermore, other viral proteins can be used as well, for example, the vesicular stomatitis virus G protein or the influenza hemagglutinin [35]. The BEVS can yield protein at levels comparable to those achieved in

bacteria or yeast, but its ability to perform complex post-translational modifications (e.g. glycosylation) is greater, and thus it is a better system for producing complex VLPs [23]. The major limitation of using an insect cell system is the purification step, due to the coproduction of baculovirus particles, a contaminant that must be biophysically or biochemically separated from VLPs (e.g., using cesium chloride ultracentrifugation or ion exchange chromatography), as they can significantly impact production efficiency [8]. This issue can be easily overcome by blocking the formation of baculovirus particles, as has been shown previously in a study of HIV VLP production and purification [36].

In conclusion, the expression of ToBMV CP gene in BEVS enables VLP self-assembly in insect cells. The N-terminal residues 2-24 of the native ToBMV CP are not essential for self-assembly into VLPs in the BEVS and can be replaced by a different sequence with medical significance, such as an epitope from a human pathogen. The modified tymovirus CP could be used as a protein carrier without affecting the self-assembly properties of the CP and, as a consequence, epitope purification and display. Therefore, tVLPs carrying small peptides facilitate antigen purification and display for vaccine production and/or other purposes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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