



# The artificial production of viral-like particles in *Nicotiana benthamiana* suggests the pro-assembly role of the Cannabis cryptic virus RdRP

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## Abstract

The partitivirids feature an icosahedral protein coating accommodating both their dsRNA genome and RNA-dependent RNA polymerase (RdRP). This signifies that transcription and replication activities of the viral polymerase occur within the capsid, emphasizing that the viral cycle relies on polymerase incorporation. Particles lacking RdRP are defective and hence non-infectious. Encapsidation and replication are intricately linked for dsRNA viruses, to the extent that, for many of these, such as the cystovirids, the RdRP serves a dual role as a transcriptase/replicase and a pro-assembly factor, ensuring structural stability and overall capsid integrity. This work investigates if RdRP has a similar role within the capsid of Cannabis cryptic virus (CanCV), a betapartitivirus affecting *Cannabis sativa*. Utilizing reverse genetics in *Nicotiana benthamiana*, we conclusively established that RdRP expression is indispensable for CanCV's virus-like particle formation. This study enhances our understanding of CanCV encapsidation, with RdRP serving a pivotal role as a pro-assembly factor. These preliminary findings contribute to the knowledge of viral assembly within the *Partitiviridae* family.

**Keywords** *Betapartitivirus* · Capsid structural integrity · dsRNA viruses

## Introduction

The term 'cryptic' is commonly used to describe numerous viruses within the *Partitiviridae* family (Boccardo et al. 1987). These multipartite cytoplasmatic viruses can infect their hosts persistently without inducing apparent symptoms, in a relationship that can be defined as symbiotic (Roossinck 2010). This viral family includes five genera that differ in host specificity: members of the genera *Alphapartitivirus* and *Betapartitivirus* infect both plants and fungi, members of the genus *Gammapartitivirus* exclusively

infects fungi, those belonging to the genus *Deltapartitivirus* are found only in plants, and members of the genus *Cryspovirus* infect protozoa (Vainio et al. 2018). A recently discovered potential species of *Partitiviridae*, the *galbut virus*, has been surprisingly found to be common in wild populations of *Drosophila melanogaster* and *Aedes aegypti*, suggesting its prevalence in these insect populations (Cross et al. 2020, 2023). In plants, these viruses maintain a low viral titer and cannot be transmitted through mechanical means, grafting, or by vectors. However, they exhibit efficient transmission through seeds. When both parents are carriers, all offspring seedlings inherit the virus (Boccardo et al. 1987). In fungi, transmission depends on hyphal anastomosis and sporogenesis (Vainio et al. 2018). These transmission characteristics result from the absence of a viral protein responsible for movement (Vainio et al. 2018). Consequently, transmission can only take place during the division of host cells (Vainio et al. 2018).

The viral genome comprises two monocistronic double strand (ds)RNA fragments encoding the RdRP and the coat protein (CP). For some partitivirids, satellite dsRNA segments are sometimes found, although no replicative

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or structural function has been assigned to them (Compel et al. 1999; Kim et al. 2005). With a few exceptions (Shimura et al. 2022), these viruses do not encode silencing suppressors. Therefore, they are believed to rely on alternative strategies, such as coupling the replication process with encapsidation, to counteract the host's gene silencing defense mechanism. Viral RdRP replicates and transcribes the dsRNA genome within the virion, ensuring that the dsRNA is never exposed to the host cytoplasmic DICER proteins. Semiconservative transcription results in the retention of the newly synthesized plus strand RNA (ssRNA(+)) inside the virion, while the template ssRNA(+) exits in the cytoplasm (Buck 1978). It either serves as a template for translation or becomes incorporated into new assembling viral particles, where RdRP utilizes it to reconstruct the dsRNA genome. Purified particles demonstrate *in vitro* replication activity (Pan et al. 2009), indicating the presence of RdRP molecules within the virion, presumed to interact with CPs through non-covalent bonds and to be anchored in proximity to one of the pores on the viral particle's surface (Nibert et al. 2014). Viral particles are non-enveloped with T = 1 icosahedral symmetry, they range in size from 25 to 43 nm in diameter (Vainio et al. 2018). The capsid is formed by 120 CP units, arranged as 60 dimers, and dimeric surface protrusions are frequently observed. Diamond shaped CP tetramers (dimers of dimers) occur as intermediate of encapsidation (Vainio et al. 2018). Such structural features are also found for Picobirnavirus core capsids (Duquerroy et al. 2009) suggesting a shared evolutionary lineage, distinct from other dsRNA viruses (Tang et al. 2010). Within the *Partitiviridae* family, capsids exhibit significant variations in size and, to some extent, in shape. In fact, some gammapartitiviruses and betapartitiviruses share similar yet distinguishable structures. For instance, excluding surface protrusions, the primary capsid shells of gammapartitiviruses have smaller outer diameters, thinner average profiles, and more angular shapes, along with smaller interior-cavity volumes (Ochoa et al. 2008; Tang et al. 2010). Furthermore, the protruding domains of their coat protein dimers form two-fold-symmetrical arches, contrasting with the two fold-symmetrical "butte-like" structures formed by betapartitivirus CP dimers (Ochoa et al. 2008; Tang et al. 2010). Recent research has demonstrated that transient expression in *Nicotiana benthamiana* can serve as an efficient tool for the production and study of partitiviridae Virus-Like Particles. By transiently expressing the CP of the deltapartitivirus pepper cryptic virus 1 (PCV1), it was possible to characterize a highly disordered and hyper-variable region within the capsid protrusions of the virus that is not required for VLP assembly (Byrne et al. 2021). This finding leads to the hypothesis that this hyper-variable region may have a role in the symbiotic relationship

between deltapartitiviruses and various dicotyledonous hosts (Byrne et al. 2021). Another inherent deduction from the results is that the virus's RdRP does not have structural roles, as the transient expression of CP alone is sufficient for VLP formation. This suggests, for deltapartitiviruses, at least for PCV1, RdRP inclusion within the capsid must occur through a mechanism separate from capsid formation.

With this work, our aim is to unravel the formation of particles of the betapartitivirus *Cannabis cryptic virus* (CanCV), which is phylogenetically distant from PCV1. First identified in European *Cannabis sativa* plants a few years ago (Ziegler et al. 2012), it has since been detected in nearly every European variety tested without inducing any evident symptoms (Miotti et al. 2023; Righetti et al. 2018). Through the transient expression of its coding RNAs in *N. benthamiana*, we intend to examine the role of RdRP in the formation of CanCV VLPs, comparing this process with well-known models of other dsRNA viruses.

## Materials and methods

### VLPs purification and TEM observation and SDS page

Viral particles of CanCV were purified from 40 g of hemp infected leaves or patch area of agroinfiltrated *N. benthamiana* according to the protocol used by Turina et al. (2007). Visible fractions were isolated from a linear 7–50% (w/v) sucrose gradient and subjected to new pelleting after ultracentrifugation at 235'000 x g for 2 h. The resulting pellets were ultimately resuspended in 30 µl of RNase-free water.

Two µl of the purification were used to visualize the presence of any VLPs with a TEM (Philips CM 10) after uranyl acetate staining (0.5–2%). Grids were prepared with the "drop" method (Bozzola and Russell 1999). Additionally, 10 µl of the purified VLPs were subjected to SDS-PAGE on a 12% polyacrylamide gel (Acrylamide/Bis-acrylamide 37.5/1) followed by Coomassie blue staining to analyze the detectable protein components.

### RNA extraction

RNA was extracted from CanCV particles purified from leaves of infected *C. sativa* and *N. benthamiana* patch area using phenol:chloroform:isoamyl alcohol (25:24:1) phase extraction method. Extracted RNA was then precipitated by adding two volumes of absolute ethanol with sodium acetate at a final concentration of 0.3 M. Subsequently, the resulting RNA pellets were resuspended in 50 µl of RNase-free water.

## Cloning of CanCV ssRNA1(+) and ssRNA2(+) into pJL89 binary vector

Full-length cDNA of both CanCV RNAs was synthesized using the RT Superscript™ IV kit from Thermo Fisher Scientific, following the manufacturer's protocol with a modification involving the denaturation of the dsRNAs-primer mix at 99°C for 5 minutes. Sequence-specific reverse primers (CanCV\_3'UTR\_RdRP\_R and CanCV\_3'UTR\_CP\_R) were employed (Table 1), and the resulting single-stranded cDNAs were then amplified using the LongRange PCR kit from Promega, following the manufacturer's instructions. We utilized primers designed to extend 5' and 3' of CanCV cDNAs with SmaI restriction site sequences (CanCV\_RdRP\_SmaI F/R and CanCV\_CP\_SmaI F/R). PCR products meeting the expected size of 2397 bp for RNA1 coding the

RdRP and of 2266 for RNA2 coding the CP were isolated and purified using the Wizard SV gel and PCR clean-up system kit (Promega) and then digested with SmaI restriction enzyme (FastDigest from Thermo Fisher Scientific). After DNA purification from the agarose gel, the CanCV double-stranded cDNA fragments were ligated to the pJL89 binary vector (Delbianco et al. 2013) with T4 DNA ligase (Thermo Fisher Scientific). Blunt-end vector was generated through PCR amplification using the Phusion High-Fidelity PCR Kit (Thermo Fisher Scientific) with pJL89\_Full\_R and pJL89\_Full\_F primer using 10 ng of circular Dam-methylated plasmid as a template. Prior to gel purification of the amplicon with the Wizard SV gel and PCR clean-up system kit from Promega, the pJL89 circular plasmid was digested by DpnI (FastDigest from Thermo Fisher Scientific). After electroporation of *Escherichia coli* MC1022 cells, PCR

**Table 1** List of utilized primers that includes the nucleotide sequences. For the PCR primer pairs, the expected lengths of the amplicons and the annealing temperature are also provided

Primer name	Sequence 5'→3'	Couple of primers used for PCR	Annealing temperature used (°C) - Expected size of the amplicon (bp)
CanCV_RdRP_SmaI_F	AAACCCGGGAGATTTTCTAAAGC GCCCGG	CanCV_RdRP_SmaI_F/CanCV_RdRP_SmaI_R	52°C - 2415 bp
CanCV_RdRP_SmaI_R	AAACCCGGGATATAAAATTTGCAAA ATCAACG	CanCV_CP_SmaI_F/CanCV_CP_SmaI_R	50°C - 2284 bp
CanCV_CP_SmaI_F	AAACCCGGGAGATTTTCTAAAGC GCCCGG	pJL89_Full_F/pJL89_Full_R	55°C - 4707 bp
CanCV_CP_SmaI_R	AAACCCGGGCTTAAAGTAGAAAA TAACGTTA	CanCV_RdRP_F/pJL89_R	55°C - 770 bp
pJL89_Full_F	GGGTCCGGCATGGCATCTCCACC	CanCV_CP_F/pJL89_R	55°C - 802 bp
pJL89_Full_R	CCTCTCCAAATGAAATGAACTTCC	CanCV_RdRP_T7_F/CanCV_3'UTR_RdRP_R	60°C - 2413 bp
CanCV_RdRP_F	ATCGCATCTCCCAATTCATC	CanCV_5'UTR_RdRP_F/CanCV_RdRP_T7_R	62°C - 2413 bp
pJL89_R	TCGGGGAAATTCGAGCTCTCCC	CanCV_CP_T7_F/CanCV_3'UTR_CP_R	60°C - 2282 bp
CanCV_CP_F	CAATGCCATGAAATCACTCG	CanCV_5'UTR_CP_F/CanCV_CP_T7_R	62°C - 2282 bp
CanCV_RdRP_T7_F	TAATACGACTCACTATAGCGCCCG CGCCTTATTTA	Tag/CanCV_RdRP_R	55°C - 222 bp
CanCV_3'UTR_RdRP_R	ACGACCGTTTCAAAAAGTAGGTG	Tag/CanCV_CP_R	55°C - 421 bp
CanCV_RdRP_T7_R	TAATACGACTCACTATAGACGACC GTTTCAAAAAGTAGGTG	CanCV_RdRP_F/CanCV_RdRP_R	55°C - 202 bp
CanCV_5'UTR_RdRP_F	CGCCCCGCGCCTTATTTA	CanCV_CP_F/CanCV_CP_R	55°C - 525 bp
CanCV_CP_T7_F	TAATACGACTCACTATAGGATTTT CTAAAGCGCCCCG		
CanCV_3'UTR_CP_R	TGTTTGTCGGAAAAGGAGGC		
CanCV_CP_T7_R	TAATACGACTCACTATAGTGTTTGT CGGAAAAGGAGGC		
CanCV_5'UTR_CP_F	GATTTTCTAAAGCGCCCCG		
CanCV_tag_RdRP_F	TGGACCCGGGATAAATTTGAATCG CATCTCCCAATTCATC		
CanCV_tag_CP_F	TGGACCCGGGATAAATTTGATCTC GAGCTACTCCCAATTTCA		
CanCV_RdRP_F	ATCGCATCTCCCAATTCATC		
CanCV_RdRP_R	GGCTTGAGTCCATTTTCAGG		
Tag	TGGACCCGGGATAAATTTGA		
CanCV_CP_R	GGCTTGAGTCCATTTTCAGG		
CanCV_Cp_F	CAATGCCATGAAATCACTCG		

screening of the recombinant colonies was performed to ensure the correct orientation of the plasmid fragments in order for the pJL89 plasmids to promote the expression of CanCV ssRNA(+) strands, referred to as pJL89\_RdRP and pJL89\_CP, respectively. This was accomplished using forward primers CanCV\_RdRP\_F or CanCV\_CP\_F (Table 1) in combination with the reverse primer pJL89\_R (Table 1). Colony PCRs were carried out using the GoTaq® G2 Flexi DNA Polymerase kit from Promega. Plasmids were purified with PureYield™ Plasmid Miniprep System (Promega) and then Sanger sequenced. Subsequently, pJL89\_RdRP and pJL89\_CP were electroporated into *Agrobacterium tumefaciens* strain GV3101 (Holsters et al., 1980).

### ***N. benthamiana* agro-infiltrations**

The desired agro-clones were cultivated in Luria-Bertani (LB) medium with appropriate antibiotics (Rifampicin 50 µM and Kanamycin 100 µM) for an overnight incubation at 28 °C. Saturated liquid cultures were then pelleted and resuspended in MES buffer containing 10mM MgCl<sub>2</sub>, 10mM 2-[N-morpholino] ethane sulfonic acid, and 200µM acetosyringone until a 600 nm optical density of 0.6 was attained. Different Agro-mixes were prepared with a 1:1:1 or 1:1 volume ratio, as detailed in Table 2. Fourteen-day-old *N. benthamiana* plants were agro-infiltrated with a needleless syringe and then grown with constant temperature and illumination (photoperiod 16/8 H day/night; temperature 22/18°C day/night; hygrometry 70%) for 7 days post infiltration (dpi), before patched area samplings.

### **Run-off in vitro transcription with CanCV PCR-amplified DNAs as a templates**

Templates for the in vitro transcription of both negative and positive CanCV ssRNAs were generated via PCR using 10 ng of purified pJL89\_RdRP and pJL89\_CP as templates. The following pairs of primers were used: CanCV\_RdRP\_T7\_F with CanCV\_3'UTR\_RdRP\_R for ssRNA1(+); CanCV\_RdRP\_T7\_R with CanCV\_5'UTR\_RdRP\_F for ssRNA1(-); CanCV\_CP\_T7\_F with CanCV\_3'UTR\_CP\_R for ssRNA2(+) and CanCV\_CP\_T7\_R with CanCV\_5'UTR\_CP\_F for ssRNA2(-). Primer details and PCR annealing conditions are listed in Table 1. PCR products were purified using the PCR

clean-up system kit from Promega and eluted in a volume of 25 µL of nuclease-free water. After quantification using a nanodrop (Thermo Fisher), 500 ng of each template was used for in vitro transcription with the RiboMAX™ Large Scale RNA Production System (Promega), in a volume of 20 µL, following the manufacturer's instructions.

### **Strand specific RT-PCR with tagged primers**

Complementary DNAs (cDNAs) of the CanCV genomic ssRNAs(-) were synthesized using tagged primers (CanCV\_tag\_RdRP/CP\_F), with a 5' end 20-nucleotide tag, unrelated to the virus (TGGACCCGGGATAAATTTGA). The reaction was carried out with 1 µL of particle purified RNAs using ImProm-II™ Reverse Transcriptase (Promega) following the manufacturer's instructions with modifications involving the denaturation of the dsRNAs-primer mix at 99 °C for 5 min, and the cDNA synthesis temperature that was set at 42 °C for 1 h. To remove the tagged primers from the reaction, the reverse transcription product was purified using the PCR clean-up system kit from Promega and eluted in a volume of 50 µL of nuclease-free water. Following purification, 2 µL of the purified product was used as a template for PCR reactions with GoTaq Flexi G2 (Promega). These PCR reactions utilized the TAG primer in combination with either the CanCV\_RdRP\_R or CanCV\_CP\_R primer to detect ssRNA1(-) and ssRNA2(-), respectively. The reactions were conducted according to the manufacturer's instructions. The primers used and RT and PCR annealing temperatures are listed in Table 1. To confirm the absence of any residues of tagged primers in the purified reverse RT product, PCRs using only the reverse primers were conducted. Additionally, to assess the specificity of the assay, it was also performed on the in vitro transcripts of positive and negative ssRNAs of CanCV. For the latter, 0.5 µL of the transcription product was used without the removal of the template DNA and performing RT step following manufacturer's instructions, including the initial nucleic acid denaturation at 70 °C for 5 min (supplementary Fig. 1). Avoiding DNA denaturation, M-MLV reverse transcriptase is prevented from utilizing ssDNAs as templates (Gerard and D'Alessio 1993).

## **Results**

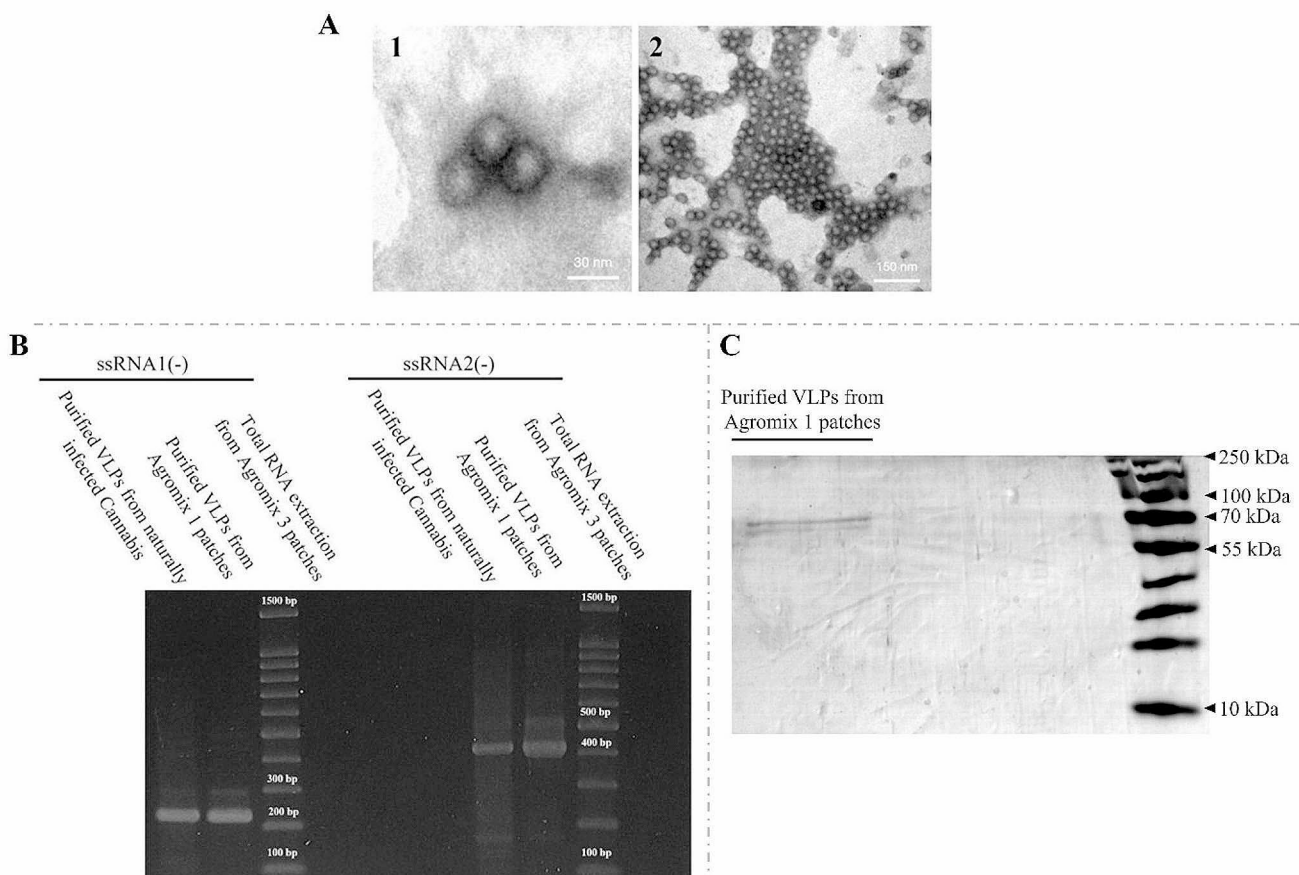
The potential role of CanCV RdRP in capsid assembly was investigated using a reverse genetics approach. By employing Agro-clones expressing the two viral ssRNA(+), transient and separate expression of these components allowed to assess whether VLP formation was dependent on functional

**Table 2** Different liquid culture mixes of *A. tumefaciens* transformed with pJL89\_RdRP, pJL89\_CP and pBIN\_p19\_TBSV used during the agroinfiltration experiments in *N. benthamiana*

Agromix	pJL89_RdRP	pJL89_CP	pBIN_p19_TBSV
1	X	X	X
2		X	X
3			X

complementation between RdRP and the structural protein CP. For this purpose, viral particles of CanCV were isolated from 40 g of infected *C. sativa* leaves. The genomic RNA extracted from these particles served as a template for the construction of the recombinant plasmids pJL89\_RdRP and pJL89\_CP. In these plasmids, the complete sequence of the two viral genomic segments was inserted between the double 35S expression promoter and the hepatitis delta virus ribozyme sequence to eliminate the polyadenylate tail from the transgenic RNAs expressed in the nucleus. This approach was taken based on the findings of Rigetti et al. (2018), which confirmed the absence of polyadenylation at the 3' ends of the CanCV wild-type genomic segments. The simultaneous transient co-expression of ssRNA1(+) and ssRNA2(+) in *N. benthamiana* leaf agro-patches (Agromix 1, Table 2) did not result in the production of purified and observable VLPs under electron microscopy. This outcome was attributed to the sense PTGS induced by the transgenes (Parent et al. 2015) indicating that neither CanCV CP nor RdRP exhibits PTGS suppression activity

in *N. benthamiana*. To address this limitation, the agroclone pBIN\_p19\_TBSV, expressing the silencing suppressor p19 from tomato bushy stunt virus (TBSV) (Peltier et al. 2012), was added to the infiltrating Agromix (Agromix 2, Table 2). After 7 dpi, in the agro-infiltrated areas, VLPs with sizes and morphological characteristics similar to those extracted from naturally infected hemp plants were successfully isolated (Fig. 1A1 and A2). SDS-PAGE analysis confirmed the presence of a protein with dimensions congruent to CanCV CP (74.88 kDa) (Fig. 1C). The slight visible band shift from the expected size could be attributed to post-translational modifications or altered detergent binding that modify CP electrophoretic properties (Rath et al. 2009; Shi et al. 2012). RdRP was not detectable, as observed in other studies on natural partitoviridae particles, where the presence of viral RdRP is presumed to be in quantities of a few molecules per particle and has been indirectly detected through in vitro enzymatic activity assays (Pan et al. 2009). A qualitative analysis of the genomic content within these particles, conducted using negative-strand-specific RT-PCR targeting



**Fig. 1** Observation of CanCV particles and analysis of protein and genomic content. TEM observation of purified particles from naturally infected *C. sativa* plants (A1) or *N. benthamiana* leaf patches infiltrated with Agromix 3 (A2). Electrophoretic analysis of the RT-PCR strand specific for ssRNA1(-) and ssRNA2(-) (B). The reactions

were performed on RNA extracts from purified particles obtained from naturally infected *C. sativa* or *N. benthamiana* leaf patches infiltrated with Agromix 3. Additionally, reactions were conducted on total RNA extracts from Agromix 3 *N. benthamiana* leaf patches." SDS page migration of Agromix 1VLPs protein content (C)

CanCV RNA1 and RNA2, revealed the presence of these ssRNAs within the VLPs (Fig. 1B). Negative PCR amplification conducted with the primer pairs CanCV\_CP\_F / CanCV\_CP\_R and CanCV\_RdRP\_F / CanCV\_RdRP\_R confirmed the lack of Agroclone-derived T-DNAs in the purified particles. These results indicate that the RdRP likely converted the ssRNA(+) strands into dsRNA within CanCV VLPs. However, it's important to specify that this analysis does not provide a quantitative assessment of ssRNA(-) relative to the purified VLPs, and the precise proportion of VLPs that encapsulate fragments of the bipartite virus genome remains to be determined. However, the expression of ssRNA2(+) alone, decoupled from the expression of RdRP, did not result in the purification of TEM-observable VLPs, indicating the necessity of the viral polymerase for their formation.

## Discussion

In the context of reverse genetic studies, it is common practice to employ in vitro system (Le and Müller 2021; Asensio-Cob et al. 2023) or non-natural host organisms as bioreactors for viral particles (Bragard et al. 2000; Abrahamian et al. 2020; Yang et al. 2021), rather than the natural hosts of the investigated virus. Notably, *N. benthamiana* has been extensively employed in numerous studies to produce Virus-Like Particles (VLPs) from various viruses, including those of animal origin, for vaccines or nanocarrier development. (D'Aoust et al. 2008; Smith et al. 2023). A result, *N. benthamiana* can be used for the expression of both wild-type and mutated structural viral components, facilitating the characterization of encapsidation mechanisms in a diverse spectrum of viruses, including the partitiviridae. In our investigation of complete virion formation, we have delved into the mechanisms characterizing this process, with a specific focus on the retention of RdRP molecules in viral particles of dsRNA viruses. The literature provides insights into various mechanisms for incorporating RdRPs, offering a clear perspective on the intricate dynamics of virion assembly. For instance, some viruses, such as yeast L-A virus, express their polymerase in the form of a fusion protein with the capsid protein (gag-pol), which is subsequently incorporated into viral particles as a minor structural component (Icho and Wickner 1989). However, in most cases, dsRNA viruses establish direct CP-RdRP interactions to anchor the replicase to the inner surface of the capsid. This interaction is essential for the activation of RdRP, which ensures the replication/transcription of the viral genome only once it is packaged inside the capsid (Patton et al. 2006).

Recently, exceptions to this previously assumed general model have been observed, particularly with the human picobirnavirus (hPBV). In hPBV, the RdRP appears to be enzymatically active without the presence of CP as a cofactor, and its packaging inside the virion does not seem to depend on a direct interaction with CP but rather on an interaction with the viral genome. The mechanism ensuring the structural integrity of the capsid of dsRNA viruses is also controversial, as experiments have shown that hPBV VLPs can form in the absence of RdRP expression (Collier et al. 2016). However, for the cystoviridae bacteriophage  $\Phi 6$ , it has been demonstrated that the polymerase has a role in procapsid assembly, accelerating the self-assembly process and stabilizing the compact conformation of the CP shell (Sun et al. 2018). Regarding partitiviridae viruses, the mechanism is still far from being fully understood. Experiments conducted in *N. benthamiana* have shown that the deltapartivirus PCV-1 can assemble VLPs solely through the expression of its CP without RdRP. Nonetheless, the potential role of the ssRNA(+) CP genomic molecule cannot be ruled out, as it is expressed as a messenger RNA by the agro-clone 35S cassette, even if it is depleted of the UTRs at the 5' and 3' ends. In our preliminary study, we delved into the capsid assembly mechanisms of another partivirus, specifically one belonging to the genus *Betapartivirus*. Our findings suggest that the formation of the capsid in CanCV is contingent upon the expression of its RdRP. It is important to note that the potential formation of VLPs consisting solely of capsid proteins cannot be definitively ruled out, as their quantity may fall below the limit of detection of our particle purification and visualization methodology. Nevertheless, our results undeniably demonstrate that the coordinated co-expression of CP and RdRP in *N. benthamiana* is associated with a remarkably more efficient production of CanCV like particles. Based on our findings, we cannot definitively confirm whether the presence of genomic RNA (+) is essential for the assembly of VLPs. However, it is apparent that the sole presence of genomic RNA (+) is not sufficient to effectively guide the translated CPs into the cytoplasm to complete nucleation and the formation of the capsid envelope. This is highlighted by the absence of VLP formation in the presence of CanCV's CP alone, despite its expression through transient agro-mediated transformation and the production of genomic RNA (+) as messenger RNA by the cellular RNA polymerase II. Although the strain-specific RT-PCR used to detect the negative strands of genomic dsRNAs within the purified particles cannot give us an indication of the amount of VLPs that retain the viral genome, it has determined that at least some of them possess negative-strand RNA, presumably part of the dsRNAs synthesized by the RdRP within the capsid. In vitro encapsidation studies of the segmented dsRNA blue tongue virus, a member of

the *Reoviridae* family, have revealed that the formation of VLPs relies on the simultaneous presence of all ten genomic ssRNA(+) strands, along with the CP, and the non-structural proteins RdRP and RNA capping enzyme (CAP) (Lourenco and Roy 2011). It is conceivable that a fine mechanism that orchestrates the nucleation and assembly of the capsid, ensuring the preservation of genomic, structural, and functional integrity to minimize the formation of defective and non-infectious particles. Indeed, other members of the *Reoviridae* family, such as rotaviruses, are believed to assemble the CP shell around RdRP/CAP/ssRNA(+) complexes. This process is initiated by the non-covalent interaction between RdRP and the disordered N-terminal extensions protruding internally from the CP capsid. It is speculated that subsequent CP-CP homotypic contacts are then enhanced and stabilized by further ssRNA(+) interactions, ultimately leading to the assembly of the capsid inner core. Similar mechanisms may be suggested for CanCV, even if our data lack the capability to quantify the proportion of purified particles possessing genomic dsRNA. Indeed, transient expression of ssRNA1(+) and ssRNA2(+), stabilized by the expression of TBSV p19, ensures an adequate cytoplasmic presence of genomic ssRNAs (+). These, co-assisted by RdRP, may initiate the capsid nucleation process. Such speculations will remain unverified unless the presence of ssRNA(+) and RdRP in each purified particle from *N. benthamiana* is demonstrated. Future comparative experiments between VLPs purified from transiently transformed *N. benthamiana* and naturally infected *C. sativa* should employ sensitive protein identification and quantification techniques, such as liquid chromatography with tandem mass spectrometry. This approach will indicate the presence of RdRP in purified particles and provide the stoichiometric ratio between CP and RdRP, and therefore between VLPs and RdRP. The ability to finely quantify viral CPs will indeed help define the absolute quantity of purified particles in a solution and associate it with the quantification of genomic ssRNA(+).

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s42161-024-01628-w>.

**Author contributions** M.D.A. and N.M. conceived the study and experiments. N.M., M.D.A. and D.B. performed the experiments. M.D.A., N.M., A.P., P.C. and C.R. wrote the manuscript.

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## Declarations

**Conflict of interest** The authors declare no conflict of interest.

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