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Cylindrical inclusion protein of chilli veinal mottle virus promotes elevated viral titers of pod pepper vein yellows virus

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Abstract

Mixed viral infections occur commonly in field plants. Such co-infections can lead to elevated titers of one or both viruses, potentially exacerbating symptom severity. When *Nicotiana benthamiana* was infected with chilli veinal mottle virus (ChiVMV, a potyvirus) and pod pepper vein yellows virus (PPeVYV, a polerovirus), there were enhanced titers of PPeVYV compared with a single infection. Immunological studies showed that ChiVMV facilitated the invasion of PPeVYV into mesophyll tissues, but did not enable the mechanical transmission of PPeVYV between plants. A three-dimensional tomography study of cells infected by the two viruses demonstrated that the spherical virions of PPeVYV were distributed within ChiVMV pinwheel inclusion bodies. Overexpression of the ChiVMV cylindrical inclusion (CI) protein enhanced the titers of PPeVYV suggesting that the CI protein acts as a co-chaperone during PPeVYV infection.

Keywords Mixed viral infections, Chilli veinal mottle virus, Pod pepper vein yellows virus, Cylindrical inclusion

Background

Pepper vein yellows disease (PeVYD) is a significant threat to pepper crops, causing symptoms such as interveinal yellowing, stunting, and leaf rolling in

Capsicum frutescens plants. The disease is caused by various viruses belong to the genus *Polerovirus* in the family *Solemoviridae*, including a newly-identified recombinant virus named pod pepper vein yellows virus (PPeVYV; *Polerovirus PPEVYV*) discovered in Wenshan City, Yunnan Province, China (Zhao et al. 2021). Poleroviruses are phloem-restricted and have isometric virus particles with diameters ranging from 24 to 26 nm (Sömera et al. 2021). Their genomes have seven open reading frames (ORFs), designated as ORF0 through ORF5 and ORF3a, which are predicted to encode proteins P0 to P5 and P3a, respectively (Murakami et al. 2011; Smirnova et al. 2015).

Mixed infections of poleroviruses and potyviruses occur frequently, creating a synergistic interaction manifested by enhanced titers of the polerovirus which is then able to invade mesophyll tissues (Savenkov and Valkonen 2001; Yang et al. 2021a). Potyviruses (members of the genus *Potyvirus* in the family of *Potyviridae*) possess genomes that encode a polyprotein which is cleaved into

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10 functional proteins by three self-encoded proteases. An additional small ORF, PIPO, is formed from RNA polymerase slippage during viral genome replication (Yang et al. 2021b). A unique characteristic of all potyvirids (members of the family of *Potyviridae*) is their ability to induce pinwheel-shaped inclusion bodies formed by cylindrical inclusions (CI) in the cytoplasm of infected cells (Yeh and Gonsalves 1985; Sorel et al. 2014). The CI protein has multiple functions including ATP binding and RNA helicase activities (Fernández et al. 1995; Kadaré and Haenni 1997) and is thus involved in viral replication, intercellular movement, and in counteracting plant resistance (Abdul-Razzak et al. 2009; Sorel et al. 2014; Deng et al. 2015; Movahed et al. 2017; Wu et al. 2022). Although the functions of CI are generally well-known, interactions between the CI protein and poleroviruses have not been previously explored.

Our previous study demonstrated that plants infected with PPeVYV alone did not display noticeable vein yellowing but pronounced interveinal yellowing occurred when co-infected with pod pepper vein yellows virus-associated RNA (PPeVYVaRNA), a tombusvirus-like RNA (Peng et al. 2021). It is noteworthy that PPeVYVaRNA was detected by RT-PCR in only 8 of the 16 PPeVYV-infected samples, suggesting that this tombusvirus-like RNA may not be the only critical components of field infections. Indeed, chilli veinal mottle virus (ChiVMV; *Potyvirus capsivenamaculae*), chilli ringspot virus (ChiRSV; *Potyvirus capsianuli*), and tomato zonate spot virus (TZSV; *Orthospovirus tomatoszoneae*), have also been identified in the samples collected. The role of potyviruses in enhancing the accumulation of PPeVYV transcripts therefore warrants further investigation.

In this study, we investigated the impact of co-infection by ChiVMV (a potyvirus) on the titers of PPeVYV (a polerovirus) and characterized the interaction between these viruses using transmission electron microscopy (TEM) and electron tomography (ET). Additionally, we examined the systemic expression of the CI protein to elucidate its role in PPeVYV infection. Our findings indicate that ChiVMV facilitates the invasion of PPeVYV into mesophyll tissues but that it does not promote mechanical transmission between plants. Notably, spherical virions of PPeVYV were found distributed within the pinwheel inclusion bodies. Furthermore, the CI protein of ChiVMV appears to assist PPeVYV infection in enhancing viral titers.

Results

Effect of coinfection with ChiVMV on titers of PPeVYV in *N. benthamiana*

PPeVYV is a novel recombinant polerovirus that causes vein yellowing symptoms on the leaves of *C. frutescens*

and which can be detected at low titers without symptoms in the uppermost leaves of inoculated young *N. benthamiana* plants. Transcriptome sequencing indicated co-infection with potyviruses (ChiVMV and ChiRSV), orthospoviruses (TZSV), and associated RNAs (PPeVYVaRNA) alongside PPeVYV in the plants where PPeVYV was first discovered (Peng et al. 2021; Zhao et al. 2021). To assess the synergistic effect of potyvirus infection on PPeVYV titers, we used a full-length cDNA infectious clone of ChiVMV developed in our previous study. Experimental treatments included ChiVMV, PPeVYV, and co-infection with ChiVMV and PPeVYV (MI), with pCB301 serving as control. Vectors were electroporated into *Agrobacterium tumefaciens* cells and delivered to *N. benthamiana* plants.

At 7 days post-inoculation (dpi), vein yellowing symptoms were clearly observed on the non-inoculated leaves of the ChiVMV and MI treatment groups, but no symptoms were evident in the pCB301 and PPeVYV groups (Fig. 1a). RT-qPCR analysis confirmed that the titers of PPeVYV in the MI treatment were nearly 75-fold higher than those observed in the PPeVYV treatment alone, while the titers of ChiVMV did not differ significantly between the ChiVMV and MI treatments (Fig. 1b). Western blot analysis corroborated these findings, showing the presence of PPeVYV-P3 in MI-infected leaves but not in the leaves infected solely with PPeVYV (Fig. 1c). Immunoprinting of mesophyll tissue from non-inoculated leaves across the four groups, stained using antibodies to PPeVYV coat protein (Anti-PPeVYV-P3) and ChiVMV coat protein (Anti-ChiVMV-CP) revealed that leaves inoculated with pCB301 and ChiVMV lacked any stained cells using anti-PPeVYV-P3 (Fig. 1d I–II). In leaves co-inoculated with ChiVMV and PPeVYV, cells infected with PPeVYV were present in relatively small clusters (Fig. 1d IV), whereas only a few cells were infected with PPeVYV in leaves infected solely with PPeVYV (Fig. 1d III). Overall, co-infection with ChiVMV significantly increased the titers of PPeVYV.

Attempted mechanical transmission of co-infecting PPeVYV and ChiVMV

To analyze the distribution of PPeVYV in systemically infected leaves co-inoculated with ChiVMV and PPeVYV, we examined ultrathin sections from four groups using transmission electron microscopy (TEM), focusing on both mesophyll and vascular tissues. In PPeVYV-infected leaves, spherical virus particles were observed only in the vascular tissues (Fig. 2). Conversely, in leaves co-inoculated with ChiVMV and PPeVYV, spherical particles of PPeVYV and pinwheel inclusion bodies characteristic of ChiVMV were observed interspersed in both mesophyll

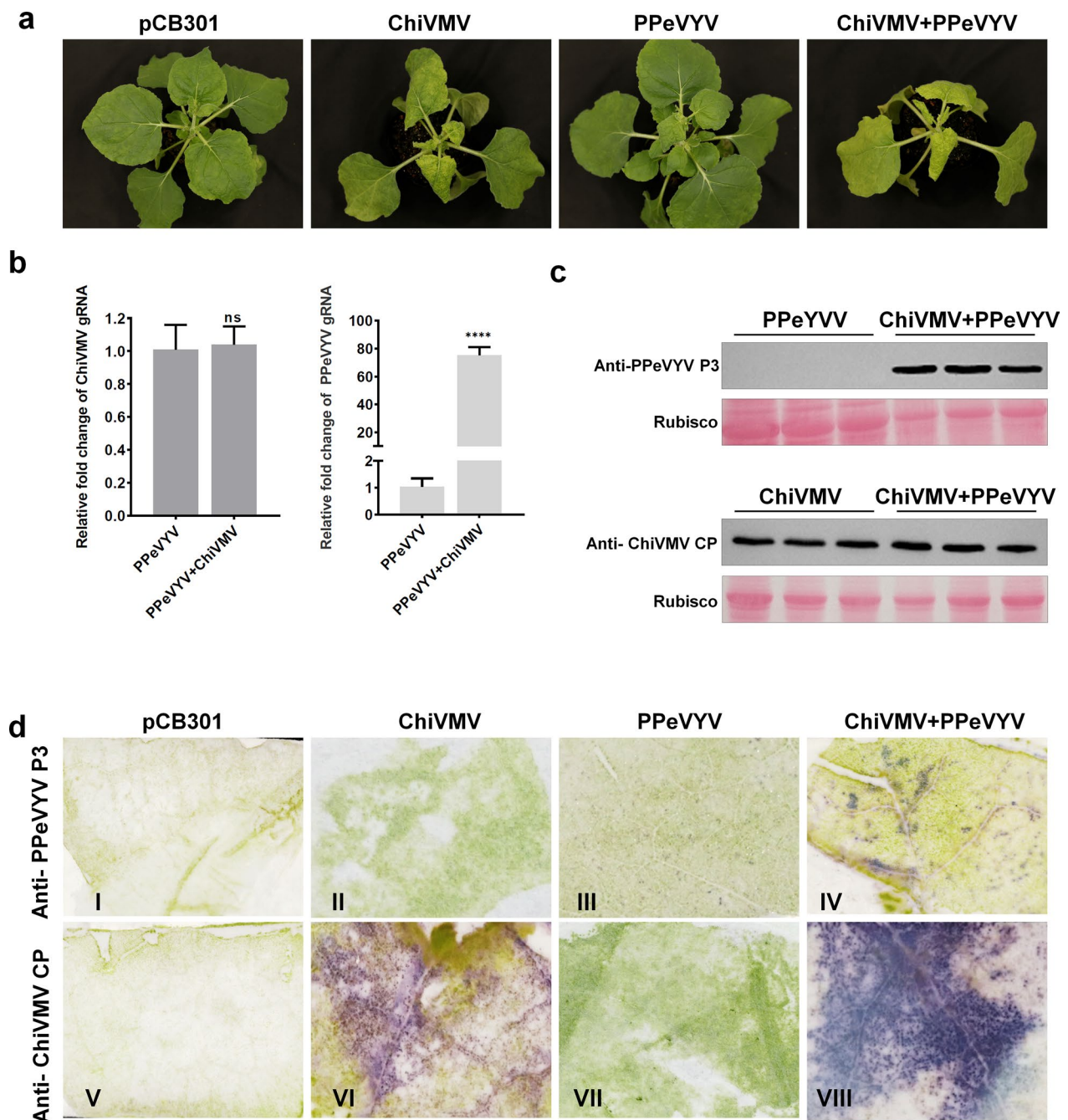


Fig. 1 Symptoms and viral accumulation in co-infection of PPeVYV and ChiVMV in *Nicotiana benthamiana*. **a** Symptoms observed in plants infected with PPeVYV, ChiVMV or co-infected with PPeVYV and ChiVMV. **b** Viral titers of PPeVYV and ChiVMV in infected plants, as determined by RT-qPCR. Three biological replicates were included and one representative is shown. **c** Western blot analysis confirming the presence of viral proteins in non-inoculated leaves of *N. benthamiana* plants. **d** Immunoblots of non-inoculated leaves from PPeVYV, ChiVMV, and mixed-infected (MI) plants, stained with anti-PPeVYV-P3 or anti-ChiVMV-CP antibodies

and vascular tissues. This suggests that ChiVMV may aid PPeVYV in invading mesophyll tissues.

To investigate whether co-infection facilitates mechanical transmission, healthy plants were sap-rubbed with infectious PPeVYV, ChiVMV, or a mixture of both.

After 5 dpi, vein yellowing symptoms appeared on non-inoculated leaves of plants inoculated with ChiVMV or the ChiVMV + PPeVYV mixture (Additional file 1: Figure S1a). Further analysis detected ChiVMV in treated plants, while PPeVYV was not found in any treatment

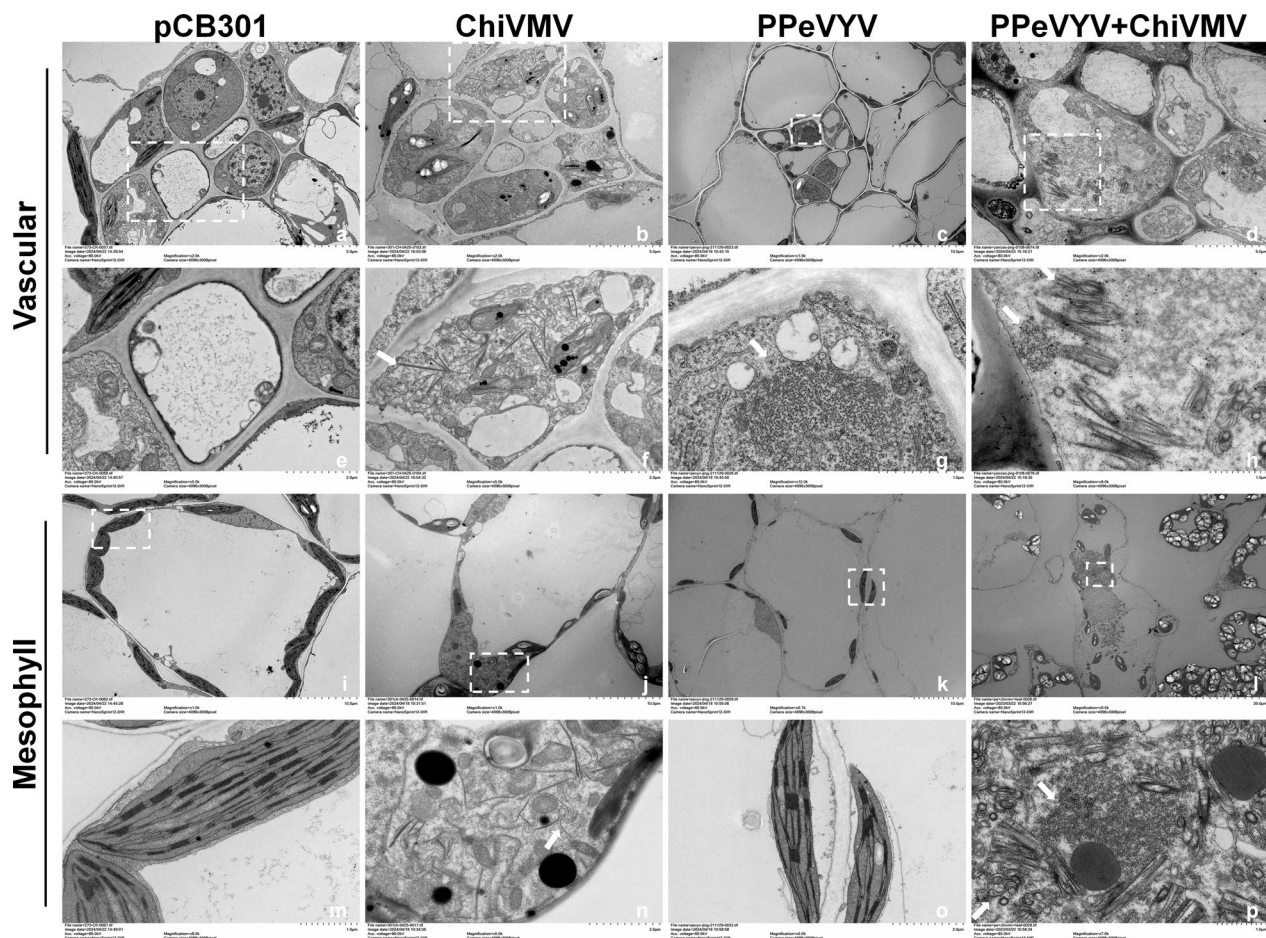


Fig. 2 Transmission electron micrographs showing the ultrastructure of virus-infected leaf cells. The distribution of spherical virions and pinwheel inclusion bodies in vascular tissues (**a–h**) and mesophyll cells (**i–p**) of pCB301 and virus-infected leaves

group (Additional file 1: Figure S1b, c). These results indicate that while ChiVMV aids PPeVYV in invading mesophyll tissues, it does not enable PPeVYV to be mechanically transmitted between plants.

Co-infection dynamics in *N. benthamiana*: interaction of poleroviruses and potyviruses

Infection of *N. benthamiana* with both poleroviruses and potyviruses results in increased polerovirus titers. The HC-Pro in potyviruses is known to inhibit antiviral defense responses in plants but fails to mitigate the phloem restriction characteristic of poleroviruses (Savenkov and Valkonen 2001). An examination of TEM images shows that spherical virions are distributed around the pinwheel inclusion bodies (Fig. 2h); however, such aggregation into clusters occurs only in PPeVYV-infected cells (Fig. 2g). To characterize this effect, we reconstructed the three-dimensional (3D) tomogram of cellular components from 200 nm section, facilitating the observation of the spatial arrangement

of the pinwheel inclusion bodies along with the spherical virions. We focused on a clustered region of mixed-infected (MI) cells for tomographic analysis, which revealed that the spherical virions were arranged on the surface of pinwheel inclusion bodies in a regular pattern, as observed in transverse sections (TS) (Fig. 3a–f; Additional file 3: Movie S1). Longitudinal sections (LS) also demonstrated that the spherical virions were distributed irregularly within the pinwheel inclusion bodies (Fig. 3g–l; Additional file 4: Movie S2), in contrast to the distribution observed in cells infected solely with PPeVYV or ChiVMV (Additional file 1: Figure S2; Additional file 5: Movies S3 and Additional file 6: Movies S4). The slices from the MI samples provided clearer insights, indicating that the spherical virions were likely associated with the pinwheel inclusion bodies (Fig. 3). The 3D data extracted from the tomogram effectively illustrated the relationship between the virions and the pinwheel inclusion bodies.

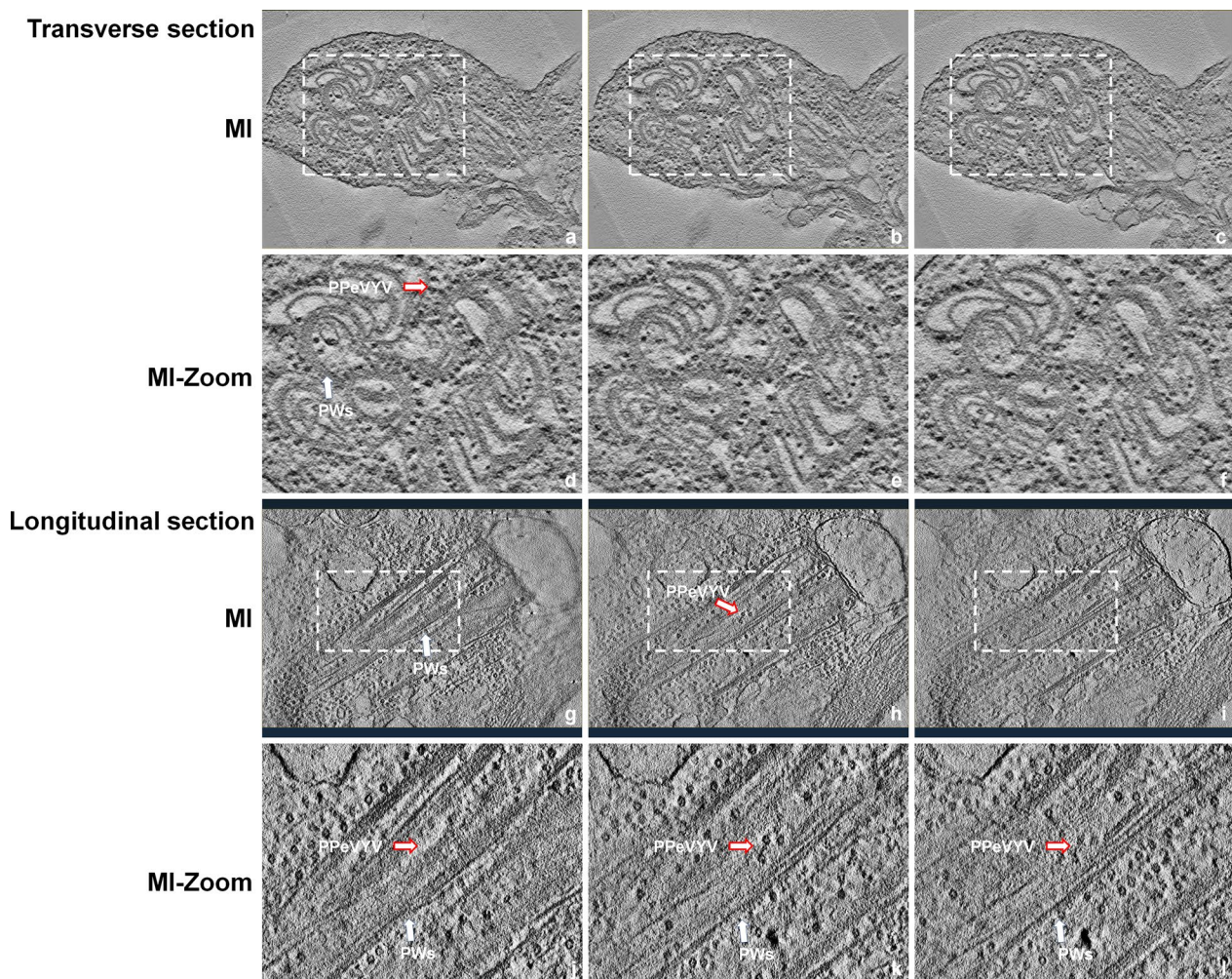


Fig. 3 Electron tomography showing the ultrastructure of MI-infected leaves. **a–f** Transverse section (TS) analysis and **g–l** Longitudinal section (LS) analysis illustrate the relationship between the virions of PPeVYV and the pinwheel inclusion bodies (PWs) of ChiVMV. **a–f** The thickness of the 3D map of MI (TS) slices along the z-axis were 90 nm, 113 nm, and 139 nm, respectively. **g–l** The thickness of the 3D map of MI (LS) slices along the z-axis were 136 nm, 164 nm, and 178 nm, respectively

Role of the CI protein of ChiVMV in enhancing PPeVYV viral titers

To assess whether the CI protein from ChiVMV could augment PPeVYV infection, we developed systemic expression vectors (TRV-CI). Systemic expression vectors were inoculated via *Agrobacterium*-mediated infiltration, with TRV-00 serving as a control. At 7 days

post-inoculation (dpi), both TRV-CI and TRV-00 infected plants were inoculated with PPeVYV through *Agrobacterium*-mediated infiltration (Fig. 4a). At 12 dpi, the P3 protein of PPeVYV was easily detected in TRV-CI plants (particularly in vein tissues), whereas it was not observed in TRV-00 infected plants (Fig. 4b). Additionally, RT-qPCR results indicated that the viral titers of

(See figure on next page.)

Fig. 4 Viral titers of PPeVYV in leaves systemically overexpressing ChiVMV CI. **a** Symptoms observed in *N. benthamiana* plants inoculated with PPeVYV from TRV-00 or TRV-CI infected plants at 12 days post-inoculation (dpi). **b** Western blot analyses confirming the presence of PPeVYV P3 or ChiVMV CI proteins in the infected leaves. N: Negative, Healthy plants; P: Positive, PPeVYV- or ChiVMV-infected plants. **c** RT-qPCR analyses confirming the presence of PPeVYV viral RNAs in the infected leaves of *N. benthamiana* plants. Three biological replicates were included and one representative is shown. **d** Confocal imaging of B2-GFP in PPeVYV or pCB301 infected leaves, showing the CI-mCherry or mCherry localization to VRCs of PPeVYV

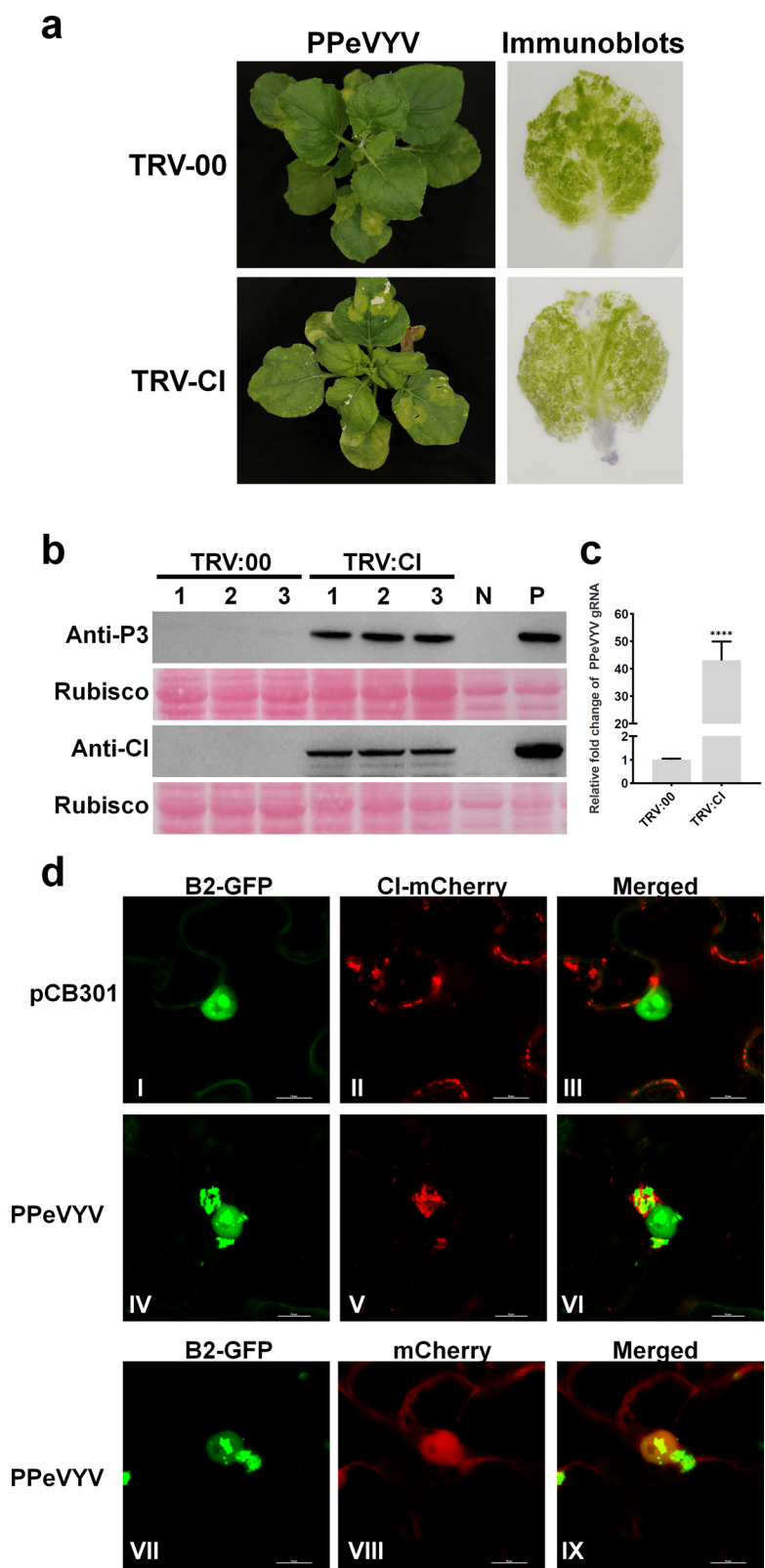


Fig. 4 (See legend on previous page.)

PPeVYV in TRV-CI plants were approximately 43-fold higher than those in TRV-00 infected plants (Fig. 4c). To investigate the interaction between CI and PPeVYV, we used B2-GFP-transgenic *N. benthamiana* for analysis. In these transgenic plants, the GFP-fused double-stranded RNA-binding domain of the B2 protein enables visualization of viral replication complexes (VRCs) (Monson et al. 2018). In B2-GFP transgenic plants, B2-GFP was localized in both the nucleus and cytoplasm, with notable enrichment observed in the nucleoli (Fig. 4d I–III). At 48 h post-inoculation (hpi) in PPeVYV-infected cells, B2-GFP formed aggregates at the periphery of the nucleus, indicating the presence of VRCs associated with PPeVYV (Fig. 4d IV). Furthermore, CI-mCherry co-localized with the VRCs of PPeVYV in the leaves infected with PPeVYV (Fig. 4d IV–VI), whereas no co-localization was observed in pCB301-infected leaves (Fig. 4d I–III). Additionally, the empty vector mCherry did not exhibit co-localization with the VRCs (Fig. 4d VII–IX). These findings suggest that the CI protein of ChiVMV enhances the accumulation of PPeVYV.

Discussion

Coevolution with associated RNAs or viruses, along with mixed infections involving these entities, can lead to increase titers of poleroviruses and support their movement from phloem to mesophyll cells (Sanger et al. 1994; Hoffmann et al. 2001; Ryabov et al. 2001; Savenkov and Valkonen 2001; Wintermantel 2005; Srinivasan and Alvarez 2007; Peng et al. 2021; Zhao et al. 2021). Poleroviruses have the ability to coexist with tombusvirus-like associated RNAs (tlaRNAs), potyviruses, umbraviruses, or satellite RNAs, which results in enhanced titers of poleroviruses and more severe disease symptoms (Sanger et al. 1994; Pruss et al. 1997; Ryabov et al. 2001; Savenkov and Valkonen 2001; Wintermantel 2005; Mo et al. 2011; Peng et al. 2021). Mixed infections of potyviruses and poleroviruses occur frequently in field plants (Wamaita et al. 2018; Yang et al. 2021a; Li et al. 2024). Potyviruses elevate the titers of the poleroviruses and promote their migration into mesophyll cells as we also found in this study. Despite the fact that the potyviral HC-Pro protein increases PLRV titers, it failed to overcome its phloem limitation (Savenkov and Valkonen 2001). Thus, for poleroviruses to penetrate mesophyll cells, the collaboration of other viral suppressors and cell-to-cell movement proteins is essential. In our study, we provide novel evidence that the CI protein of ChiVMV serves as a viral factor responsible for increasing PPeVYV titers although its mechanistic role in promoting the spread of PPeVYV into mesophyll cells remains to be determined.

Genetic and cellular biological studies have established that P3N-PIPO, CI, P3, and CP are critical for the

cell-to-cell movement of potyviruses (Revers and García 2015). This research indicates that the spherical virions of PPeVYV are distributed within pinwheel inclusion bodies. CI is a multifunctional protein typical of potyvirids; it is known to form pinwheel inclusion bodies as part of the VRC and plays a role in facilitating cell-to-cell movement by interacting with CP (Fernández et al. 1995; Abdul-Razzak et al. 2009; Sorel et al. 2014; Deng et al. 2015). Our findings suggest that while CI can enhance the viral titers of PPeVYV, significant penetration into mesophyll cells was not observed. It appears that CI must interact with CP as docking point in PD for intercellular movement of viral replication vesicles. The CI does not possess a PD localization signal peptide and instead relies on P3N-PIPO for targeting to PD (Wei et al. 2010). This implies that potyvirus proteins involved in cell-to-cell movement may facilitate the spread of poleroviruses into mesophyll tissues. Notably, the polerovirus capsid consists of two forms of coat protein: the major P3 protein and the minor P3/P5 protein, which results from a translational readthrough (RTD) of P3. Both P3 and the RTD are critical for the viral infection cycle. Mutants lacking part or all of the PLRV P5 domain were able to move into and infect nonvascular mesophyll tissues when the source-sink relationship in *Solanum sarrachoides* was altered, with the progeny virus subsequently becoming mechanically transmissible (Peter et al. 2009). This finding suggests that P5 of poleroviruses may be involved in maintaining the viral phloem limitation. Phloem limitation is likely a result of co-evolution with host plants, allowing the virus to evade RNA-mediated decay while enhancing transmission by vector aphids. Further investigation is needed to determine whether the viral proteins of umbraviruses or potyviruses can interact with RTD to promote the invasion of poleroviruses into mesophyll tissues.

Conclusion

In the field, mixed infections involving poleroviruses and potyviruses occur frequently, resulting in a synergistic interaction that is reflected by elevated titers of poleroviruses and an increased capacity to invade mesophyll tissues. The underlying mechanisms of these interactions remain inadequately defined. This study indicates that the multifunctional protein CI may enhance both the titers of poleroviruses and their movement into mesophyll tissues. Nevertheless, the extent to which CI facilitates polerovirus infection—either directly or indirectly—requires further investigation. While previous studies have established the vital role of viral particles in the long-distance dissemination of poleroviruses, the specifics of their cell-to-cell movement are still not fully elucidated. Observations from co-infected sample

slices provide valuable insights, suggesting that spherical poleroviral virions are probably associated with potyviral pinwheel inclusion bodies. This implies that virions may also be crucial for the cell-to-cell movement of poleroviruses. Therefore, clarifying the mechanisms underlying cell-to-cell movement is essential for understanding how poleroviruses could penetrate mesophyll tissues.

Methods

Plants growth conditions

N. benthamiana plants were cultivated in a greenhouse maintained at 24 °C with a relative humidity of 55%. They were subjected to a photoperiod of 16 h of light followed by 8 h of darkness.

Plasmid construction

Transient expression vectors were assembled through ligation-independent cloning. The coding sequences of the CI protein were amplified, yielding 1932 bp fragments, using KOD-plus Neo (Toyobo) with cDNA from ChiVMV-infected plants as the template. Subsequently, these fragments were inserted into the pTRV2 vectors (Additional file 2: Table S1).

Transient expression by *Agrobacterium tumefaciens*

Recombinant plasmids were transformed into the cells of *A. tumefaciens* strain C58C1 via electroporation. *Agrobacterium* cultures were grown overnight at 28 °C, harvested by centrifugation, and suspended in infiltration buffer containing 10 mM MES, 10 mM MgCl₂, and 200 μM acetosyringone (pH 5.6). The optical density was adjusted to OD₆₀₀=0.5 or other appropriate concentrations as required for different experiments. Infiltration of *N. benthamiana* leaves with *A. tumefaciens* cultures was performed using an injector without a syringe needle.

RNA extraction and quantitative real-time PCR analysis

Total RNA extraction from *N. benthamiana* was performed using TRIzol (Invitrogen, USA) according to the manufacturer's guidelines. Reverse transcription (RT) was carried out on the extracted RNA using ReverTra Ace[®] qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan), in accordance with the manufacturer's instructions. *Nbactin* served as an internal control during the RT-qPCR analysis. Reactions were conducted using the LightCycler 480 II PCR System (Roche, Switzerland), and data were analyzed following the 2^{-ΔΔCT} method (Livak and Schmittgen 2001).

Western blotting

Total proteins from the plant tissues were extracted with a lysis buffer consisting of 100 mM Tris-HCl (pH 8.8), 60% SDS, and 2% β-mercaptoethanol. Following

extraction, the proteins were resolved by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes (Amersham, GE Healthcare Life Sciences) using the Fast Wet Transfer System eBlot[™] L1 (GE Healthcare Life Sciences, Amersham, UK). The primary antibodies used for detection included anti-CP (1:5000), anti-P3 (1:1000), and anti-CI (1:5000). Membranes were visualized using the Immobilon Western Chemiluminescent HRP Substrate (Millipore, USA), in accordance with the manufacturer's protocol.

Immunohistochemical localization

In the print hybridization assay, leaves were press-blotted onto filter paper (Whatman, UK). After blocking, the leaves were incubated with primary antibodies, anti-CP and anti-P3, followed by incubation with an anti-rabbit secondary antibody (Proteintech, USA). Visualization was performed using nitrotetrazolium blue chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) buffer (Solarbio, China), according to the manufacturer's protocol.

Mechanical inoculation

Mechanical inoculation was performed by sprinkling silicon dioxide (Macklin, China) onto the leaves of *N. benthamiana*. The leaves infected with PPeVYV or both PPeVYV and ChiVMV were ground and diluted in phosphate-buffered saline (PBS). The resulting extracts were applied to *N. benthamiana* leaves via dripping, followed by gentle smearing with a cotton swab.

Virus-induced gene silencing

A. tumefaciens strains carrying pTRV1 and pTRV2 vectors (pTRV2:00, pTRV:PDS, or pTRV2-CI) were combined at a 1:1 ratio before co-infiltrating the leaves of *N. benthamiana*. After the appearance of bleaching symptoms in plants infected with TRV:PDS, those infected with TRV:00 or TRV:CI were inoculated with *A. tumefaciens* containing the cDNA infection clone of PPeVYV. Further analyses were carried out 10 days post-inoculation.

Electron tomography

Ultrathin section preparation and observation techniques were previously detailed by Jiao et al. (2024). For electron tomography (ET) analysis, 200 nm thick sections were cut by an ultramicrotome (Leica EM UC7, Germany) and mounted onto 100-mesh carbon-coated copper grids. Initially, sample sections were treated with 10 nm colloidal gold particles (Sigma, MO) for 5 min on both sides, followed by staining as described by Xie et al. (2019). Image collection involved capturing four series of images of the VP37-tubule and one series of viral components

using a JEM-2010 transmission electron microscope (TEM) (JEOL, Tokyo, Japan) at an accelerating voltage of 200 kV, recorded with a Gatan Rio 9 CMOS camera (size 2.7 k × 4 k) (Gatan, USA). The TEM specimen stage was tilted from -60° to $+60^\circ$ at 1° intervals. Images were taken with a defocus value of 6 μm at a magnification of 12,000 $\times$ and a beam density of 26 e/s $\text{\AA}^2$.

Images were processed, aligned, and reconstructed using the IMOD 'etomo' program according to the software manual. Fine alignment was achieved using twenty gold particles as fiducial markers for each tilt series, resulting in a 'residual error' value of less than 0.3. Final 3D tomograms for each tilt series were constructed by merging images with an $\text{\AA}/\text{pix}$ size of 5.49 (compression parameter 'binning=2') in 'etomo'. Dimension measurements were performed using the '3dmod' command of the IMOD program (Kremer et al. 1996), and model visualization was executed with the 'Amira' program (ThermoFisher, USA).

Data analysis

All analyses were conducted using GraphPad Prism 8.0.2 software.

Abbreviations

ChiVMV	Chilli veinal mottling virus
ChiRSV	Chilli ringspot virus
CMV	Cucumber mosaic virus
CP	Coat protein
GFP	Green fluorescent protein
HC-Pro	Helper-component proteinase
MP	Movement protein
ORFs	Open reading frames
PeVYD	Pepper vein yellows disease
PEMV-2	Pea enation mosaic virus-2
PLRV	Potato leafroll virus
PPeVYV	Pod pepper vein yellows virus
PPeVYVaRNA	Pod pepper vein yellows virus-associated RNA
PTGS	Post-transcriptional gene silencing
PVY	Potato virus Y
RdRp	RNA-Dependent RNA Polymerase
RT	Reverse transcription
TEM	Transmission electron microscopy
tlaRNAs	Tombusvirus-like associated RNAs
TZSV	Tomato zonate spot virus

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s42483-025-00339-z>.

Additional file 1: Figure S1. Mechanical transmission of mixed infection with PPeVYV and ChiVMV in *Nicotiana benthamiana*. **Figure S2.** Electron tomography showing the ultrastructure of leaves infected by ChiVMV or PPeVYV.

Additional file 2: Table S1. Primers used in this study.

Additional file 3: Movie S1. Transverse section (TS) analysis of leaves infected with MI was performed using electron tomography.

Additional file 4: Movie S2. Longitudinal section (LS) analysis of leaves infected with MI was performed using electron tomography.

Additional file 5: Movie S3. Electron tomography of leaves infected with PPeVYV was conducted.

Additional file 6: Movie S4. Electron tomography of leaves infected with ChiVMV was conducted.

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Author contributions

SB, QW, XM, FY, and JP conceived and designed the experiments. SB, QW, LW, and YM performed the experiments. JW, SW, and XM collected the samples and analyzed the data. SB, QW, FY, and JP wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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