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Identification of critical interaction sites in the coat protein of Areca palm velarivirus 1 (APV1) and its pathogenicity

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Abstract

Areca palm velarivirus 1 (APV1), a member of the genus *Velarivirus* (family *Closteroviridae*), is an economically significant pathogen associated with yellow leaf disease in Areca palm (*Areca catechu* L.), causing substantial declines in both yield and quality decline. The APV1 genome comprises 11 open reading frames (ORFs), with ORF6 and ORF7 encoding the coat protein (CP) and minor coat protein (CPm), respectively. In this study, the interaction between the APV1 CP and CPm proteins was identified by yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays. To clarify critical interaction sites, a double mutant (CP^{R211A–R212A}) was generated through site-directed mutagenesis for both Y2H and BiFC analyses. The result demonstrated that these residues were determined to be key amino acid sites. Furthermore, subcellular localization analysis revealed that the CP^{R211A–R212A} mutation did not alter the functional localization of the CP protein in *Nicotiana benthamiana* leaves, as it remained present in both the nucleus and cytoplasm. The pathogenicity of the APV1 CP protein and its mutant was investigated using the potato virus X (PVX) expression system. Experimental results showed that *N. benthamiana* plants infected with PVX-CP exhibited severe chlorosis symptoms. Intriguingly, *N. benthamiana* plants infected with PVX-CP^{R211A–R212A} showed markedly reduced chlorosis. Our findings indicated that the ²¹¹RR²¹² residues of APV1 CP are crucial for their interaction with the CPm protein and likely play a key role in the symptom formation. Moreover, this study represents the first identification of APV1 CP as a pathogenic factor, offering new insights into the molecular mechanisms underlying APV1 pathogenicity.

Keywords APV1, CP, CPm, Protein–protein interaction, Pathogenic factor

Background

Areca palm (*Areca catechu* L.) is extensively cultivated in tropical and subtropical regions of Southeast Asia. Recognized as one of the four major southern medicinal herbs in Hainan, it exhibits diverse medicinal properties (Li et al. 2021). As a key economic crop in Hainan, the Areca palm planting area and total yield accounts for over 95% of the country, making it a primary income source for local farmers (Hainan Provincial Bureau of Statistics 2023). However, Areca palm yellow leaf disease (APYLD), is one of the primary diseases that severely endanger the production of Areca palm cultivation,

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resulting in the yellowing and the death of the plant (Tan et al. 2023). Areca palm velarivirus 1 (APV1), initially identified in Hainan, China (Yu et al. 2015), is a primary pathogen associated with APYLD. The disease caused by APV1 exhibits high transmissibility, and currently, no effective control measures are available. In recent years, the expansion of Areca palm cultivation areas in China has intensified, exacerbating the outbreaks of APV1 as an epidemic viral pathogen in Hainan and resulting in substantial economic losses (Cao et al. 2021).

APV1, classified under the genus *Velarivirus* within the family *Closteroviridae*, is characterized by curved flexuous filamentous virus particles and a positive-sense single-stranded RNA genome (Fuchs et al. 2020; Liu et al. 2021). The complete genome of the APV1 WNLG1 strain comprises 17,546 nucleotides (nt) and encodes 11 open reading frames (ORFs). Notably, ORF6 and ORF7 encode the coat protein (CP) and minor coat protein (CPm), respectively (Wang et al. 2020). Members of the *Closteroviridae* family are distinguished by their dual coat protein system, where CP and CPm encapsulate the viral genomic RNA at opposite ends, with CP enveloping over 97% of the genomic RNA regions (Martelli 2019). Recent studies have indicated that these structural proteins also play crucial roles in viral transmission, replication, and cell-to-cell movement (Wei et al. 2019; Kokane et al. 2020).

Since APV1 has only been reported in Areca palm plants in Hainan, China for a relatively short period, the biological function and pathogenic mechanism of the virus are still unclear. Therefore, there are few reports on the interaction between viral protein and viral protein, as well as the interaction between viral protein and host protein. Ten host proteins were identified to interact with APV1 CP protein by Y2H screening (Xing et al. 2024). In a separate study, Chen et al. confirmed the self-interaction capability of APV1 CP through Y2H and BiFC assays (2024). We speculate that both CP and CPm are necessary components to encapsulate the APV1 RNA genome. However, whether the APV1 CP interacts with the CPm is not clear, and the mechanism of key interaction sites in the assembly of virus particles is unclear.

Viral pathogenic factors encoded by plant viruses play pivotal roles during virus-host interaction. These proteins disrupt the normal physiological functions of plants via multiple mechanisms, resulting in the manifestation of disease symptoms (Paudel and Sanfaçon. 2018). For example, the P23 protein of Citrus tristeza virus (CTV) acts as a pathogenic factor to regulate the proportion of positive-strand RNA over negative-strand RNA in the process of virus replication, which is related to the pathogenicity of the virus (Yang et al. 2021). The

C5 protein of tomato yellow leaf curl virus (TYLCV) enhances its pathogenicity by inhibiting plant post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS) to resist plant antiviral mechanisms (Zhao et al. 2022). Similarly, the CPm protein of tomato chlorosis virus (ToCV) functions as a pathogenicity factor that suppresses host RNA silencing induced by single-stranded RNA and enhances the pathogenicity of heterologous virus (Yang et al. 2023). However, the potential function of APV1 CP protein is a pathogenic factor in the process of virus and host interaction remains uninvestigated.

This study aims to clarify the interactions between APV1 CP and CPm, and CP protein is a potential pathogenic factor. To attain these research objectives, Y2H and BiFC assays were performed to systematically explore their molecular interactions between APV1 CP and CPm, and identify crucial binding residues of CP. Concurrently, the PVX heterologous expression system was used to infect *N. benthamiana* plants to evaluate the changes in symptoms caused by CP and CP^{R211A-R212A} mutant. These findings offer crucial insights into the pathogenic mechanisms of APV1 CP, laying a solid theoretical foundation for targeting viral protein interactions in disease control strategies.

Results

Gene cloning of APV1 CP, CPm, and CP^{R211A-R212A}

Total RNA was extracted from APV1-infected areca palm leaves, and reverse transcribed into first-strand cDNA. PCR amplification was performed using specific primers NC-APV1-CP-F/R, and a specific DNA band of 891 bp was detected (Fig. 1). This size was in accordance with the anticipated size of APV1 CP. Additionally, a clear DNA band of 1881 bp was amplified using primers NC-APV1-CPm-F/R (Fig. 1), corresponding to the anticipated size of APV1 CPm. Sequencing and blastn analysis confirmed that these two nucleotide sequences were identical to the CP and CPm genes of the APV1 WNLG1 strain (GenBank: MW316015.1). The APV1 CP^{R211A-R212A} mutation was introduced using overlap extension PCR. Regarding the PCR fragment of the APV1 CP^{R211A-R212A} gene, a specific DNA band sized at 891 bp was cloned (Fig. 1). Sanger sequencing verified the APV1 CP^{R211A-R212A}, confirming the intended substitutions.

Verification of the interaction between APV1 CP and CPm proteins

The results showed that all co-transformants exhibited robust growth on SD/-Trp/-Leu medium, confirming the successful transformation of each combination into yeast. Yeast cells from each combination were subsequently selected and cultured in

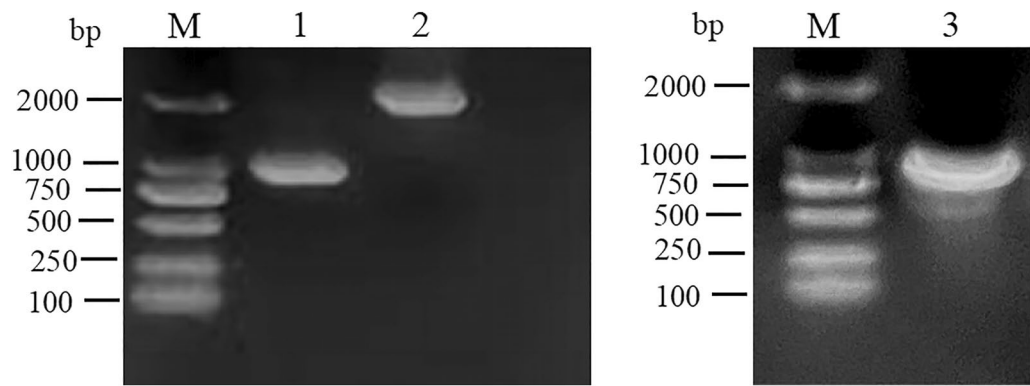


Fig. 1 PCR amplification of APV1 *CP*, *CPm*, and *CP^{R211A-R212A}* genes. M, DL2000 DNA Marker; 1, PCR amplification of APV1 *CP* gene from virus-infected Areca palm leaf; 2, PCR amplification of APV1 *CPm* gene from virus-infected Areca palm leaf; 3, PCR amplification of APV1 *CP^{R211A-R212A}* gene; N, Negative control from ddH₂O

SD/-Trp/-Leu/-His/-Ade (40 µg/mL X-α-Gal, 200 ng/mL AbA) medium for five days. Only the yeast cells from the positive control (pGADT7-T + pGBKT7-53) and the experimental group (pGBKT7-CP + pGADT7-CPm) grew on this medium, forming blue colonies. In

contrast, the negative controls failed to grow normally (Fig. 2a). Interestingly, the yeast cells from another experimental group (pGBKT7-CPm and pGADT7-CP) did not grow in SD/-Trp/-Leu/-His/-Ade (40 µg/mL X-a-Gal, 200 ng/mL AbA) medium. These Y2H results

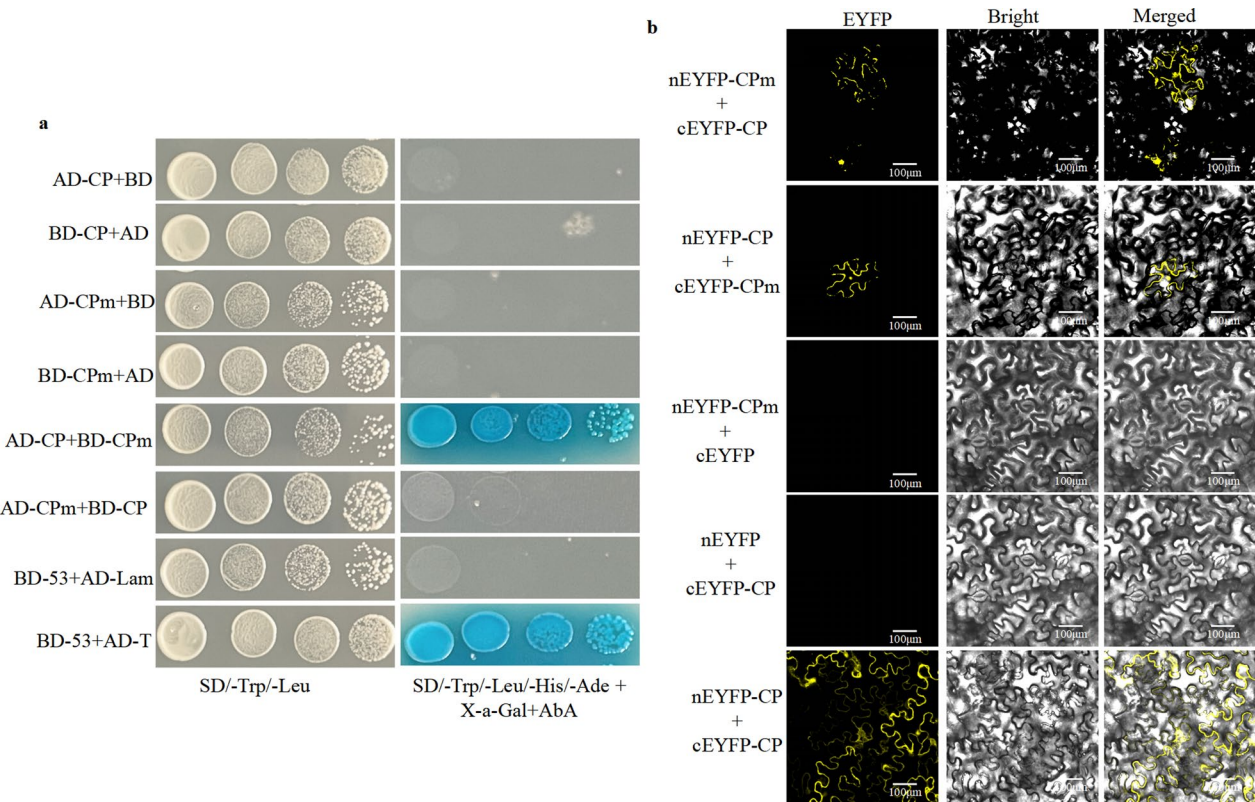


Fig. 2 Identification of the APV1 CP protein interaction with CPm. **a** Identification of the APV1 CP protein interaction with CPm by Y2H assay. Experimental group, BD-CP and AD-CPm combination, BD-CPm and AD-CP combination; Negative control, BD-CP and AD, AD-CP and BD, BD-CPm and AD, AD-CPm and BD, AD and BD-Lam; Positive control, AD-T and BD-53. **b** Identification of the APV1 CP protein interaction with CPm by BiFC assay. Experimental group, Ecc-CP and Enn-CPm, Ecc-CPm and Enn-CP; Negative controls, Ecc-CP and Enn, Ecc-CPm and Enn, Ecc and Enn-CP, Ecc and Enn-CPm; Positive control, Ecc-CP and Enn-CP; EYFP: Yellow fluorescent protein, observed at 514 nm excitation wavelength; Scale bar: 100 µm

confirmed the interaction between the APV1 CP and CPm proteins.

To further verify this interaction, BiFC assays were conducted using the experimental groups (Ecc-CP + Enn-CPm, Ecc-CPm + Enn-CP) by agroinfiltrating *N. benthamiana* leaves. The agroinfiltrated leaves were examined under laser confocal microscopy with an excitation wavelength of 514 nm, showing prominent yellow fluorescence signals in both the nucleus and cytoplasm (Fig. 2b). The yellow fluorescence was also observed in the positive control but not in the negative control group. These BiFC results corroborated the findings from the Y2H assays, thereby confirming the interaction between the APV1 CP and CPm proteins.

Key amino acid residues in APV1 CP and its interaction with CPm protein

To elucidate the critical amino acid residues of APV1 CP involved in the interaction, we performed sequence alignment and similarity analysis comparing the APV1 CP protein with CP proteins from six viruses within the *Velarivirus*. The analysis revealed that positions ²¹¹RR²¹² of the APV1 CP protein are conserved, and both residues are positively charged arginines (Fig. 3a). Consequently, we introduced mutations to change these two arginines to alanines (CP^{R211A-R212A}) and subsequently evaluated the interaction between CP^{R211A-R212A} and the CPm protein.

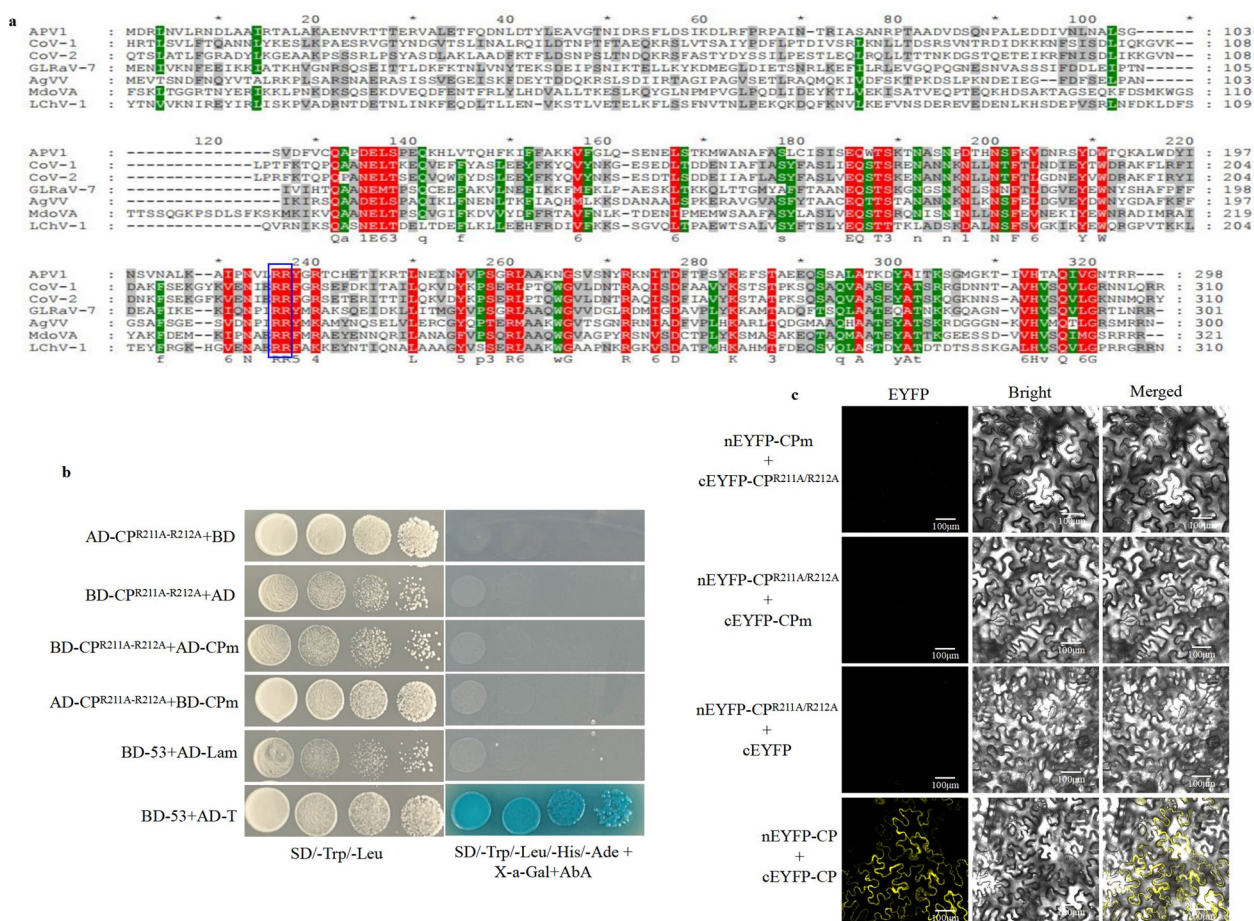


Fig. 3 Identification of key amino acids of APV1 CP protein interaction with CPm. **a** Comparison of the amino acid sequences of the APV1 CP with other CPs from Velariviruses. APV1, Areca Palm Velarivirus 1 (Genbank no. MW316015.1); CoV-1, Cordyline virus 1 (Genbank no. NC_038421); CoV-2, Cordyline virus 2 (Genbank no. NC_043453.1); GLRaV-7, Grapevine leafroll-associated virus 7 (Genbank no. NC_016436.1); AgV, Agapanthus velarivirus (Genbank no. MT533605.1); MdoVA, Malus domestica virus A (Genbank no. NC_055599.1); LChV-1, Little cherry virus 1 (Genbank no. MN131069.1). **b** Identification of key amino acids of APV1 CP protein interaction with CPm by Y2H assay. Experimental group, BD-CP^{R211A-R212A} and AD-CPm, BD-CPm and AD-CP^{R211A-R212A}; Negative control, BD-CP^{R211A-R212A} and AD, AD-CP^{R211A-R212A} and BD, AD-T and BD-Lam; Positive control, AD and BD-53. **c** Identification of key amino acids of APV1 CP protein interaction with CPm by BiFC assay. Experimental group, Ecc-CP^{R211A-R212A} and Enn-CPm, Ecc-CPm and Enn-CP, Negative control, Ecc-CP^{R211A-R212A} and Enn; Positive control, Ecc-CP and Enn-CPm; EYFP: Yellow fluorescent protein, observed at 514 nm wavelength; Scale bar: 100 μm

The Y2H results demonstrated that only the positive control group (pGADT7-T+pGBKT7-53) exhibited normal growth on SD/-Trp/-Leu/-His/-Ade medium supplemented with 40 $\mu\text{g/mL}$ X- α -Gal and 200 ng/mL AbA, eventually turning blue. In contrast, no yeast colonies were observed in the experimental groups involving pGBKT7-CP^{R211A-R212A}+pGADT7-CPm or pGBKT7-CPm+pGADT7-CP^{R211A-R212A}, indicating disruption of the original protein interaction (Fig. 3b). These findings suggest that the amino acid residues at positions ²¹¹RR²¹² of APV1 CP are crucial for CP protein interaction.

BiFC assays further confirmed these results. No yellow fluorescence was detected in the leaves of *N. benthamiana* for the experimental groups Ecc-CP^{R211A-R212A}+Enn-CPm and Ecc-CPm+Enn-CP^{R211A-R212A}. Conversely, yellow fluorescence was visible in the positive control *N. benthamiana* leaves under 514 nm excitation light, confirming the absence of interaction between CP^{R211A-R212A} and CPm. Collectively, these data identify ²¹¹RR²¹² as essential residues for CP-CPm protein interaction (Fig. 3c).

Subcellular localization of APV1 CP, CPm, and CP^{R211A-R212A} proteins

The laser confocal microscopy observation demonstrated that the green fluorescence signals from the GV1300-CP recombinant plasmid-expressed protein were localized in

both the cytoplasm and the nucleus of *N. benthamiana* cells. Similarly, the GV1300-CPm recombinant plasmid-expressed protein also exhibited green fluorescence signals in the cytoplasm and the nucleus, indicating identical intracellular distribution for both CP and CPm proteins. For the GV1300-CP^{R211A-R212A} recombinant plasmid, which was generated by mutating key residues ²¹¹RR²¹² of the CP protein, the expressed protein's fluorescence signals remained distributed in the cytoplasm and nucleus, consistent with the localization pattern of the CP protein. This suggests that the CP^{R211A-R212A} mutation does not affect the subcellular localization of the protein (Fig. 4).

Pathogenicity of APV1 CP protein and its detection

To investigate whether the APV1 CP protein functions as a viral pathogenicity determinant, this study constructed recombinant PVX-CP and PVX-CP^{R211A-R212A} vectors. These constructs were agroinfiltrated into *N. benthamiana* leaves, and symptom progression was monitored over 15 days. The positive control consisted of tobacco plants infected with PVX, while the negative control comprised healthy tobacco plants. The results indicated that systemic leaves of tobacco infected with PVX-CP exhibited mild mottling and mosaic symptoms on 7 dpi, followed by visible chlorosis on 9 dpi, and culminating severe chlorosis with leaf wrinkling by 15 dpi. In contrast, PVX-CP^{R211A-R212A} caused attenuated

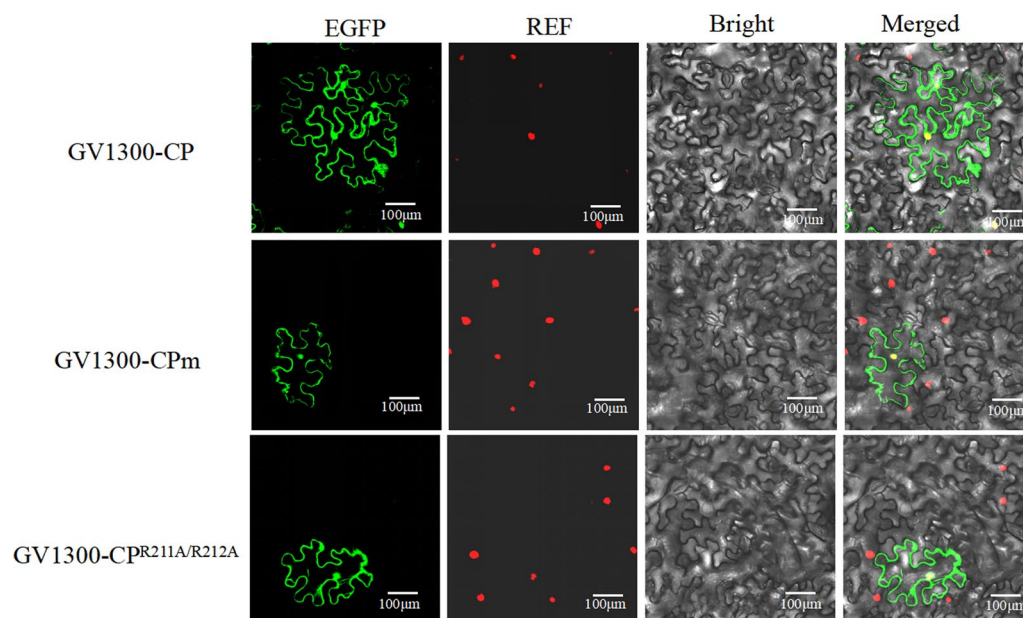


Fig. 4 Subcellular localization of APV1 CP, CPm, and CP^{R211A-R212A} proteins. CP-EGFP, CP-enhanced green fluorescent protein, observed at 488 nm wavelength; CPm-EGFP, CPm-enhanced green fluorescent protein, observed at 488 nm wavelength; CP^{R211A-R212A}-EGFP, CP^{R211A-R212A}-enhanced green fluorescent protein, observed at 488 nm wavelength; Nucleus-RFP, Nucleus-red fluorescent protein, observed at 561 nm wavelength; Scale bar: 100 μm

symptoms in tobacco plants, manifesting as mild mottling and mosaic symptoms on 7 dpi, and limited chlorosis development thereafter. Tobacco plants infected with PVX displayed mottling and mosaic symptoms on 5 dpi, but these symptoms gradually alleviated over time, becoming indistinguishable from those of healthy tobacco plants (Fig. 5a).

Systemic leaves from tobacco plants infected with PVX, PVX-CP and PVX-CP^{R211A-R212A} were collected for quantitative reverse transcription PCR (RT-qPCR) on 15 dpi. The results indicated that the transcriptional levels of the PVX CP gene varied among different vectors. The transcriptional levels of PVX-CP and PVX-CP^{R211A-R212A} were both significantly increased ($p < 0.05$) compared with that of the PVX empty vector, with 4.50 times and 3.25 times higher, respectively. However, there was no significant difference between PVX-CP and PVX-CP^{R211A-R212A} (Fig. 5b).

Systemic leaves from tobacco plants infected with PVX, PVX-CP, PVX-CP^{R211A-R212A}, and healthy controls

were collected for Western blot analysis on 15 dpi. The results revealed a specific protein band of approximately 33.8 kDa in the leaves of *N. benthamiana* infected with PVX-CP and PVX-CP^{R211A-R212A}, consistent with the expected size of the target protein (Fig. 5c). No protein bands were detected in the leaves of PVX-infected or healthy tobacco plants. These findings further confirmed that the CP protein is a critical pathogenic factor of the APV1 virus.

Discussion

The majority of viral proteins exhibit multifunctional characteristics, with the CP and CPm playing crucial roles in the viral infection and replication process (Atabekova et al. 2023; He et al. 2023). Previous studies have demonstrated that CP and CPm are involved in virus assembly and intracellular movement. For instance, CTV CP and CPm are essential for virus particle assembly, and mutations in these proteins can result in shorter particles and restricted movement (Tatineni

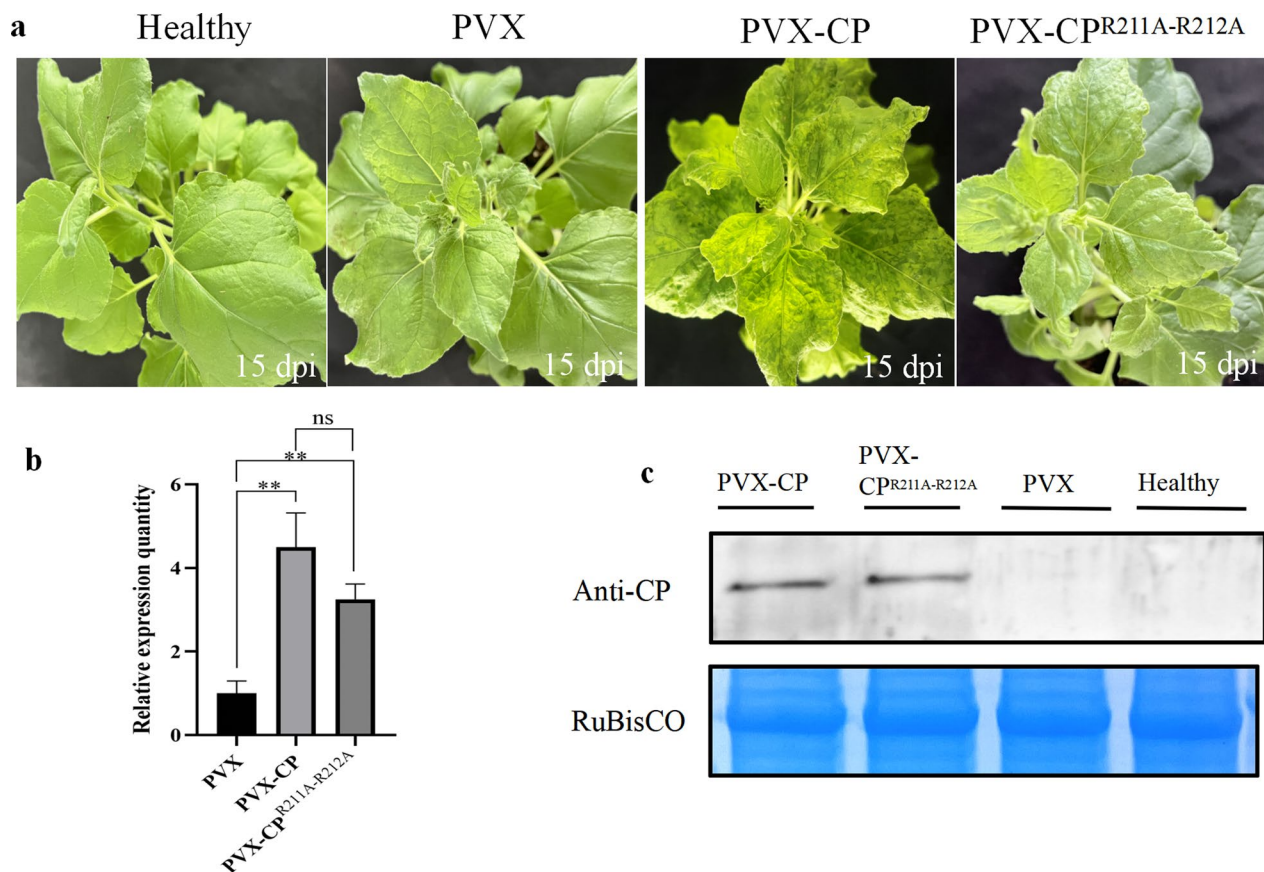


Fig. 5 Pathogenicity of PVX-CP and PVX-CP^{R211A-R212A} in *N. benthamiana*. **a** The symptoms in *N. benthamiana* plants inoculated with PVX-CP or PVX CP^{R211A-R212A} at 15 dpi. Healthy *N. benthamiana* plants as the negative controls; *N. benthamiana* plants inoculated with PVX as the positive control. **b** Transcription levels of PVX accumulation by RT-qPCR. Relative transcription levels of PVX CP gene in systemic leaves from *N. benthamiana* inoculated with PVX-CP or PVX-CP^{R211A-R212A} at 15 dpi by RT-qPCR. **c** Western blotting analysis of APV1 CP protein in systemic leaves from *N. benthamiana* plants infected with PVX-CP or PVX CP^{R211A-R212A} at 15 dpi

et al. 2010). In Beet yellows virus (BYV), CP primarily protects the viral genome, while CPm facilitates cell-to-cell movement. Mutations in either protein can impede virus movement (Alzhanova et al. 2001). Furthermore, viral proteins typically do not function independently but often require interactions with other proteins to carry out their functions. Studies have shown that Grapevine leafroll-associated virus 3 (GLRaV-3) CP interacts with HSP70h to stabilize virus particle assembly (Mostert et al. 2023), and CTV CP interacts with p33 to modulate host defense responses against viral infection (Dao et al. 2020). However, there is no existing literature on whether APV1 CP and CPm interact during the replication process. Therefore, this study utilized the Y2H and BiFC to identify the interaction between APV1 CP and APV1 CPm, revealing that this interaction is a critical factor in virus particle formation or stabilization.

AlphaFold3, developed by DeepMind, accurately predicts protein–protein and protein–biomolecule interactions, providing detailed structural insights (Wee et al. 2024). In this study, AlphaFold 3 predicted that the key interaction region between APV1 CP and CPm is located at the C-terminus of CP. Through

comparison of the amino acid sequence of APV1 CP with those of related viruses, several conserved residues were identified. After analyzing their physicochemical properties, arginines at positions 211 and 212 ($^{211}\text{RR}^{212}$) were selected for mutation to alanines, resulting in the construction of the APV1 CP^{R211A–R212A} mutant. Experimental verification confirmed that residues $^{211}\text{RR}^{212}$ are critical for the interaction, consistent with the predicted results. Structural predictions using AlphaFold3 revealed that the R211A and R212A mutation in the CP protein altered its three-dimensional architecture, thereby disrupting its interaction with CPm (Fig. 6). This computational finding was experimentally validated by site-directed mutagenesis, which demonstrated that residues $^{211}\text{RR}^{212}$ are critical for maintaining CP–CPm binding. Additionally, AlphaFold3 modelling indicated that the C-terminal domain of APV1 CP (residues 200–250) forms an α -helix interface with CPm, corroborating our mutagenesis data showing that $^{211}\text{RR}^{212}$ are essential for interaction. Notably, pGBKT7–CPm + pGADT7–CP failed to grow on selective medium, likely due to improper folding or modification induced by vector fusion, leading to reduced expression levels and

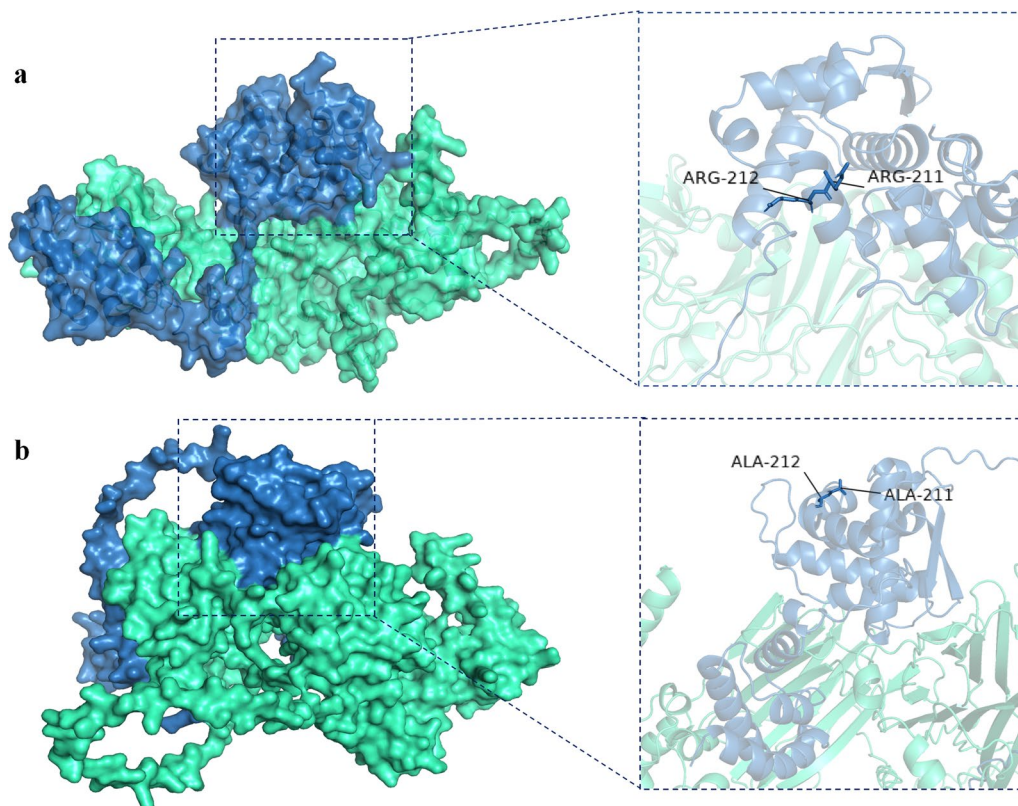


Fig. 6 Interaction prediction of three-dimensional structure between APV1 CP and CPm protein, or APV1 CP^{R211A–R212A} and CPm proteins. **a** AlphaFold3 predicts that the interaction region between the CP and CPm proteins. **b** AlphaFold3 predicts the interaction region between the CP^{R211A–R212A} and CPm proteins. Blue represents CP or CP^{R211A–R212A} protein, and green represents CPm protein

undetectable weak interactions under stringent screening conditions (Janiket et al. 2017). However, swapping the CP and CPm gene into pGBKT7-CP and pGADT7-CPm vectors resulted in robust growth on SD/-Trp/-Leu/-His/-Ade medium containing X- α -Gal/AbA, confirming specific CP-CPm interaction. To overcome Y2H system limitations, BiFC assays were also conducted, further validating the interaction between CP and CPm.

Amino acid mutations can significantly affect protein stability and have a profound impact on protein function (Álvarez-Machancoses et al. 2019). Through targeted mutagenesis experiments at residues ²¹¹RR²¹² of APV1 CP, we demonstrated that these positions form crucial sites for interaction with CPm. The sub-cellular localization of APV1 CP and CPm proteins was investigated using fluorescent protein labeling technology. The results indicated that both the individual and interactive localizations of APV1 CP and CPm are confined to the cytoplasm and nucleus. After mutation to alanines at ²¹¹RR²¹², the sub-cellular localization of APV1 CP^{R211A-R212A} remains in the cytoplasm and nucleus, suggesting that the mutation does not alter the cellular distribution of CP and CPm proteins. However, post-mutation analysis revealed no interaction between CP and CPm proteins, indicating that ²¹¹RR²¹² are critical binding sites for APV1 CP, rather than affecting protein binding through spatial distribution. To determine their relative contributions to CP stability and function, we employed two software tools for the analysis. MAESTRO (<https://biwww.che.sbg.ac.at/maestro/web>) (Laimer et al. 2015) and DUET (<http://structure.bioc.cam.ac.uk/duet>) (Pires et al. 2014) software tools predict changes in protein stability by calculating the Gibbs free energy difference ($\Delta\Delta G$, kcal/mol). In this study, single-point alanine substitutions were introduced at polar-charged residues within the APV1 CP protein. Computational analyses from both tools consistently indicated that substitutions at the ²¹¹RR²¹² dipeptide site significantly destabilized the CP structure. Compared with single-point mutations of other amino acids listed in Table 1, R211 and R212 were predicted to be consistently unstable by both software, and the mutation may affect CP protein stability or binding ability. Notably, R212A substitution exhibited a more pronounced destabilizing effect compared to that of R211A, with higher $\Delta\Delta G$ values in MAESTRO (positive values indicate destabilization) and lower $\Delta\Delta G$ values in DUET (negative values indicate reduced stability) (Table 1). These findings suggest that residue 212 plays a critical role in maintaining CP stability and function, warranting further experimental validation.

Many viral proteins have been identified as pathogenic factors, causing symptoms such as chlorosis, mosaic,

Table 1 MAESTRO and DUET predict the changes in the $\Delta\Delta G$ of the CP protein caused by single-point mutations

Substitutions	MAESTR $\Delta\Delta G_{pred}^a$	DUET $\Delta\Delta G_{pred}^b$
E114A	1.61	- 0.384
E162A	0.93	- 0.782
R211A	0.73	- 0.824
R212A	1.01	- 1.093
R215A	0.86	- 1.198
R235A	0.02	- 0.021
R247A	- 0.02	- 1.209
K258A	1.34	- 0.584
H288A	1.76	0.527

R211A and R212A were conducted in this study

^a Values of $\Delta\Delta G < 0$ mean stabilization

^b Values of $\Delta\Delta G > 0$ mean stabilization

and necrosis in host plants. Previous studies on the protein functions of various virus members within the *Closteroviridae* family highlight the critical role of the CP protein in the virus pathogenic process. For instance, the CP protein of ToCV enhanced the pathogenic symptoms during PVX-mediated infection of *N. benthamiana* (Cañizares et al. 2013). The CP protein of CTV suppresses intercellular silencing by preventing the spread of silencing signals (Dao et al. 2020). Similarly, the CP protein of Cucurbit chlorotic yellows virus (CCYV) can enhance the pathogenicity via PVX and may be a key factor directly involved in virus pathogenesis (Gan et al. 2024). Unlike some CP proteins of *Closteroviridae*, which primarily mediates virion assembly, APV1 CP exhibits dual roles in both particle formation and symptom induction, underscoring its multifunctional nature. In this study, we conducted a preliminary investigation into the pathogenicity of APV1 CP using the PVX heterologous expression system. The PVX vector, derived from potato virus X, is often used to study the pathogenicity of foreign genes (Wang et al. 2019; Mardanov and Ravin 2021). Our findings indicate that the CP protein encoded by APV1 induces more severe and prolonged symptoms in host plants compared to PVX infection. Mutation of the ²¹¹RR²¹² residues to alanines in APV1 CP alleviated pathogenic symptoms in host plants, suggesting that these residues may be key sites for the pathogenic function of APV1 CP. Further research is necessary to elucidate the mechanisms by which APV1 CP protein functions to cause pathogenic symptoms in host plants.

Conclusions

This study demonstrated the interaction between APV1 CP protein and APV1 CPm protein, with arginine residues ²¹¹RR²¹² in the CP C-terminal domain being

critical for this interaction. Subcellular localization analysis revealed that the $CP^{R211A-R212A}$ mutant retained nuclear-cytoplasmic localization, excluding mislocalization as the cause of binding disruption. Heterologous expression of APV1 CP via the PVX induced severe chlorosis and leaf malformation in *N. benthamiana*, whereas the $CP^{R211A-R212A}$ mutant significantly attenuated these symptoms, establishing APV1 CP as a key virulence determinant. This study represents the first functional characterization of APV1 CP in pathogenicity, highlighting its dual role in both virion assembly and symptom induction.

Methods

Gene cloning of APV1 CP, CPm, and mutated $CP^{R211A-R212A}$

N. benthamiana and Areca palm plants infected with APV1 were cultivated and maintained under controlled laboratory conditions. Total RNA was extracted from APV1-infected Areca palm leaves using the RNAPrep Pure Polyphenolic Plant Total RNA Extraction Kit (Tiangen, Beijing, China) after homogenization in liquid nitrogen. First-strand cDNA synthesis was performed using HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China). The APV1 CP and CPm genes were amplified by PCR using high-fidelity DNA polymerase (Cwbio, Jiangsu, China) with primer pairs NC-APV1-CP-F/R and NC-APV1-CPm-F/R (Table 2), respectively. PCR conditions were as follows: initial denaturation at 94 °C for 30 s; 35 cycles of 94 °C for 30 s, 55 °C for 40 s, 72 °C for 60 s; final extension at 72 °C for 10 min. Amplification products were verified by electrophoresis on 1% agarose gels.

To introduce R211A-R212A substitutions in APV1 CP, overlap extension PCR was conducted. Two primer pairs, APV1- $CP^{R211A-R212A}$ -1F/1R and APV1- $CP^{R211A-R212A}$ -2F/2R (Table 2), were used to generate overlapping fragments containing the desired mutations. Purified first-round PCR products (equimolar ratios, total ≤ 100 ng) were subjected to overlap extension, followed by amplification with primers NC-APV1-CP-F/R to obtain the full-length APV1 $CP^{R211A-R212A}$ mutant gene.

Construction of Y2H vectors

The purified CP and CPm genes were cloned into Y2H vectors pGADT7 and pGBKT7 using the Nimble Cloning Kit (NC Biotech, Hainan, China) according to the manufacturer's instructions. The recombinant plasmids were transformed into *Escherichia coli* DH5 α competent cells (Weidi, Shanghai, China). Positive clones were identified by colony PCR, and three colonies with correctly sized PCR products were selected for Sanger sequencing (Magen Biotech, Guangzhou, China). Verified plasmids were extracted using the HiPure Plasmid Mini Kit (Magen Biotech, Guangzhou, China), yielding recombinant plasmids named pGADT7-CP, pGBKT7-CP, pGADT7-CPm, and pGBKT7-CPm. Following this protocol, the APV1 $CP^{R211A-R212A}$ mutant gene was cloned into pGADT7 and pGBKT7 vectors, generating recombinant plasmids pGBKT7- $CP^{R211A-R212A}$ and pGADT7- $CP^{R211A-R212A}$.

PCR identification and Sanger sequencing of pGBKT7-CP/DH5 α , pGADT7-CP/DH5 α , pGBKT7-CPm/DH5 α , and pGADT7-CPm/DH5 α confirmed that

Table 2 Primers used in this study

Prime name	Primer sequence (5'-3')	Usage
NC-APV1-CP-F	AGTGGTCTCTGTCCAGTCTATGGATAGACTTAACGTAAGTAG	Nimble Cloning for CP
NC-APV1-CP-R	GGTCTCAGCAGACCACAAGTCCGCTAGTATTACCAACA	
NC-APV1-CPm-F	AGTGGTCTCTGTCCAGTCTCTATGTTAATTGAGAATAAACA	Nimble Cloning for CPm
NC-APV1-CPm-R	GGTCTCAGCAGACCACAAGTCTTTCGTGTCAAGATCTTC	
GV1300-CP-F	GGACTAGTATGGATAGACTTAACGTAAGTAG	Subcellular localization for CP
GV1300-CP-R	CGGGATCCCCGTCTAGTATTACCAACAATTGG	
GV1300-CPm-F	GGACTAGTATGTCACAATCTTATGCTTCTGAA	Subcellular localization for CPm
GV1300-CPm-R	CGGGATCCCTTCGTGTCAAGATCTTCCATG	
APV1- $CP^{R211A-R212A}$ -1F	CAAGCTTTCATGGATAGACTTAACGTAAGTAG	Overlap PCR for $CP^{R211A-R212A}$
APV1- $CP^{R211A-R212A}$ -1R	CTTGATAGTCTCATGACAAGTTGCTCCATACGCTGC	
APV1- $CP^{R211A-R212A}$ -2F	AAGGCAATCCCAACGTGTTGGCAGCGTATGGAGCA	
APV1- $CP^{R211A-R212A}$ -2R	CCTCGAGTTACCGTCTAGTATTACCAACA	
qPVX-CP-F	TACAGCCCGTGCCATAGTAGCC	RT-qPCR for PVX CP
qPVX-CP-R	GCTCTGCTGATGCCGTTGGAAT	
qNbActin-F	GGCATTTCATGAAACCATACACA	RT-qPCR for <i>N.benthamiana</i> actin
qNbActin-R	AGGACAATGTTCCGTACAGAT	

the *CP* and *CPm* fragments were accurately inserted into pGBKT7 or pGADT7 plasmids without any frameshift, nucleotide deletions or mutations. These plasmids were subsequently purified for protein–protein interaction verification using Y2H assays (Additional file 1: Figure S1a). Similarly, the *CP*^{R211A–R212A} mutation was successfully introduced into pGBKT7-*CP*^{R211A–R212A} and pGADT7-*CP*^{R211A–R212A} (Additional file 1: Figure S1b).

Construction of BiFC vectors

Following the Nimble Cloning protocol, APV1 *CP*, APV1 *CPm*, and APV1 *CP*^{R211A–R212A} genes were ligated into BiFC vectors pNC-BiFC-Ecc (Ecc) and pNC-BiFC-Enn (Enn). This yielded recombinant plasmids named Ecc-*CP*, Enn-*CP*, Ecc-*CPm*, Enn-*CPm*, Ecc-*CP*^{R211A–R212A}, and Enn-*CP*^{R211A–R212A}. Colony PCR and DNA sequencing validated the successful insertion of APV1 *CP* and *CPm* genes into pNC-BiFC-Ecc (Ecc) and pNC-BiFC-Enn (Enn) vectors without any frameshifts, deletions, or mutations. The resulting constructs were designated as Ecc-*CP*, Enn-*CP*, Ecc-*CPm*, and Enn-*CPm* for subsequent protein–protein interaction verification by BiFC assays (Additional file 2: Figure S2a). Additionally, the *CP*^{R211A–R212A} was correctly inserted into Ecc-*CP*^{R211A–R212A} and Enn-*CP*^{R211A–R212A} (Additional file 2: Figure S2b).

Construction of subcellular localization vectors

The *CP* and *CPm* genes were amplified using primers GV1300-*CP*-F/R and GV1300-*CPm*-F/R. Both the amplified products and the GV1300 vector (pCambia1300: GFP) were digested with restriction enzymes *Spe* I and *Bam*H I (Thermo Scientific, Massachusetts, America) (Chen et al. 2024). Purified target fragments and linearized vectors were ligated using T4 DNA ligase (Vazyme, Nanjing, China). The ligation products were transformed into *E. coli* DH5α, followed by colony PCR screening to identify positive clones. Three randomly selected colonies with correctly sized PCR products were subjected to Sanger sequencing. Verified plasmids were extracted using the HiPure Plasmid Mini Kit, yielding recombinant plasmids GV1300-*CP* and GV1300-*CPm*. Using the same protocol, the *CP*^{R211A–R212A} mutant gene was cloned into the GV1300 vector, resulting in the recombinant plasmid GV1300-*CP*^{R211A–R212A}.

Colony PCR and Sanger sequencing confirmed the accurate insertion of *CP*, *CPm*, and *CP*^{R211A–R212A} into GV1300-*CP*, GV1300-*CPm*, and GV1300-*CP*^{R211A–R212A} without any frameshifts, nucleotide deletions, or mutations. Furthermore, these recombinant plasmids were verified by double digestion with *Spe* I and *Bam*H I (Additional file 3: Figure S3), confirming their successful construction for subcellular localization studies.

Identification of the interaction between APV1 CP and APV1 CPm by Y2H assay

Plasmid was transformed into yeast Y2HGold competent cell (Weidi, Shanghai, China) using the PEG/LiAc method according to the manufacturer's protocol (Ur et al. 2019). For experimental groups, the following co-transformations were performed: pGBKT7-*CP*+pGADT7-*CPm*, and pGBKT7-*CPm*+pGADT7-*CP*. Negative controls included: pGBKT7-*CP*+pGADT7, pGADT7-*CP*+pGBKT7, pGBKT7-*CPm*+pGADT7, pGADT7-*CPm*+pGBKT7, and pGADT7-T+pGBKT7-Lam. Positive control: pGADT7-T+pGBKT7-53. Transformed yeast cells were plated on SD/-Trp/-Leu medium and incubated statically at 30 °C for 3–5 days. Each colony was resuspended in 100 µL sterile water, serial dilutions (undiluted, tenfold, 100-fold, and 1000-fold), and 5 µL of each dilution was spotted onto SD/-Trp/-Leu/-His/-Ade (40 µg/mL X-α-Gal, 200 ng/mL AbA) plates. Plates were incubated inverted at 30 °C for 3–5 days, and colony growth was documented photographically. All experiments were performed in triplicate to ensure reproducibility.

Identification of the interaction between APV1 CP and APV1 CPm by BiFC assay

Plasmids Ecc-*CP*, Enn-*CP*, Ecc-*CPm*, Enn-*CPm*, Ecc, and Enn were individually transformed into chemically competent GV3101 (pSoup-p19) cells (Weidi, Shanghai, China). The following co-infiltration combinations were prepared. Ecc-*CP*+Enn-*CPm*, and Ecc-*CPm*+Enn-*CP* for experimental groups. Negative controls included: Ecc-*CP*+Enn, Ecc-*CPm*+Enn, Ecc+Enn-*CP*, and Ecc+Enn-*CPm*; Positive control was Ecc-*CP*+Enn-*CP* (Chen et al. 2024). Bacterial suspensions were prepared according to Miao et al. (2022) and infiltrated into the abaxial side of *N. benthamiana* leaves for transient expression. Infiltrated plants were incubated in darkness at 25 °C for 24 h, followed by growth under 16 h light/8 h dark cycle at 25 °C for 72 h. Leaf sections from the injection sites (n=3) were excised and analyzed for protein interaction using a confocal laser microscope with a 514 nm excitation wavelength.

Identification of the key amino acid sites of APV1 CP interaction with APV1 CPm

The APV1 *CP* was compared with the *CP* proteins from the other six viruses in the genus *Velarivirus*. It was found that the two arginines at positions 211–212 (²¹¹RR²¹²) of the APV1 *CP* protein were highly conserved within the genus. These residues are hypothesized to be key sites for interaction with the *CPm* protein. Therefore, we co-transformed pGBKT7-*CP*^{R211A–R212A}+pGADT7-*CPm*, or pGBKT7-*CPm*+pGADT7-*CP*^{R211A–R212A} into

Y2HGold as the experimental groups; Negative controls included pGBKT7-CP^{R211A-R212A} + pGADT7, pGADT7-CP^{R211A-R212A} + pGBKT7, and pGADT7-T + pGBKT7-Lam; Positive control was pGADT7-T + pGBKT7-53. We further used BiFC to verify that the ²¹¹RR²¹² is the key site for the CP interaction with the CPm. The methods are the same as described above. Experimental groups included Ecc-CP^{R211A-R212A} + Enn-CPm, and Ecc-CPm + Enn-CP^{R211A-R212A}; Negative control was Ecc-CP^{R211A-R212A} + Enn; Positive control was Ecc-CP + Enn-CPm.

Subcellular localization analysis of APV1 CP, CPm, and CP^{R211A-R212A} proteins

GV1300-CP, GV1300-CPm, GV1300-CP^{R211A-R212A} and GV1300 were transformed into chemically competent GV3101 cells (Weidi, Shanghai, China) according to the manufacturer's instructions. According to Carvalho et al. (2022), GV1300-CP/GV3101, GV1300-CPm/GV3101, GV1300-CP^{R211A-R212A}, and GV1300/GV3101 were respectively injected into the abaxial side of *N. benthamiana* leaves to enable their transient expression with three biological replicates. Tobacco plants were kept at 25 °C for 24 h in darkness, then grown in a growth chamber at 16 h light/8 h dark cycles at 25 °C for 3 days. Finally, leaf sections from the injection sites were excised and observed under a laser confocal microscope with a 488 nm excitation wavelength for subcellular localization analysis.

Inoculation of *N. benthamiana* plants with PVX-CP and PVX-CP^{R211A-R212A}

PVX, PVX-CP, and PVX-CP^{R211A-R212A} recombinant vectors were transformed into chemically competent GV3101(pSoup) cell using the same method as above. PVX/GV3101(pSoup), PVX-CP/GV3101(pSoup), and PVX-CP^{R211A-R212A}/GV3101(pSoup) were respectively injected into the abaxial side of *N. benthamiana* leaves to infect the plants. Tobacco plants were placed in darkness at 25 °C for 24 h, then cultivated in a growth chamber at 6 h light/8 h dark cycles at 25 °C. Disease development was observed over 15 days, and photos were taken for documentation. All experiments were performed in triplicate to ensure reproducibility.

RT-qPCR analysis of PVX-CP and PVX-CP^{R211A-R212A} transcription in *N. benthamiana* plants

Total RNA was extracted from the systemic leaves using RNeasy Pure Polyphenolic Plant Total RNA Extraction Kit. The concentration and purity of extracted RNA were assayed by NanoDrop Lite Plus (Thermo Scientific, Massachusetts, America), and 1000 ng of RNA was used

to synthesize the first strand of cDNA for RT-qPCR using HiScript III All-in-one RT SuperMix Perfect for qPCR (Vazyme, Nanjing, China) following the manufacturer's instructions. RT-qPCR was conducted using the Hieff UNICON® Universal Blue qPCR SYBR Green Master Mix (Yeasen, Shanghai, China) on the CFX Opus 96 Real-Time PCR System (Bio-rad, California, America). The *N. benthamiana actin* (*NbActin*) gene was selected as an internal control for the assays. The primers of qPVX-CP-F/R for RT-qPCR are listed in Table 2. All the gene transcription data were collected in three technical replicates for each biological condition tested to assess reproducibility, and the relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method for analysis (Zhao et al. 2021).

Western blot analysis of APV1 CP and CP^{R211A-R212A} expression in *N. benthamiana* plants

To confirm that APV1 CP is the pathogenic factor causing lesions in *N. benthamiana* plants infect with recombinant virus, systematic leaves from tobacco plants infected with PVX, PVX-CP, and PVX-CP^{R211A-R212A} for 15 days, a minimum of three biological replicates, were collected. After grinding the leaves in liquid nitrogen, NP-40 buffer (Beyotime Biotechnology, Shanghai, China) was added at a ratio of 2:1 (g/v=g/mL). Western blot analysis was performed according to the method described by Dashprakash et al. 2019. The primary antibody was APV1 CP polyclonal antiserum (1:1000), and the secondary antibody was horseradish peroxidase-labeled goat anti-mouse IgG (H+L) (1:5000) (Cwbio, Jiangsu, China). Total protein from healthy tobacco leaves served as the negative control.

Abbreviations

APV1	Areca palm velarivirus 1
APYLD	Areca palm yellow leaf disease
BiFC	Bimolecular fluorescence complementation
BYV	Beet yellows virus
CCYV	Cucurbit chlorotic yellows virus
CP	Coat protein
CPm	Minor coat protein
CTV	Citrus tristeza virus
Ecc	PNC-BiFC-Ecc
Enn	PNC-BiFC-Enn
GLRaV-3	Grapevine leafroll-associated virus 3
GV1300	PCAMBIA1300: GFP
nt	Nucleotide
ORF	Open reading frame
PTGS	Post-transcriptional gene silencing
PVX	Potato virus X
RT-qPCR	Quantitative reverse transcription PCR
TGS	Transcriptional gene silencing
ToCV	Tomato chlorosis virus
TYLCV	Tomato yellow leaf curl virus
Y2H	Yeast two-hybrid

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s42483-025-00354-0>.

Additional file 1: Figure S1. Colony PCR identification of Yeast two-hybrid recombinant vectors.

Additional file 2: Figure S2. Colony PCR identification of BiFC recombinant vectors.

Additional file 3: Figure S3. Enzyme digestion verification of GV1300 recombinant plasmids.

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Author contributions

HW and NY designed experiments. RM and TC performed the experiments and analyzed the data. RM and NY performed analyzed the data. RM wrote the manuscript, XF, JW, MH, HW, and NY revised the manuscript. All authors read and approved the final manuscript.

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The authors declare that they have no competing interests.

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References

- Álvarez-Machancoses Ó, De Andrés-Galiana EJ, Fernández-Martínez JL, Kloczkowski A. Robust prediction of single and multiple point protein mutations stability changes. *Biomolecules*. 2019;10:67. <https://doi.org/10.3390/biom10010067>.
- Alzhanova DV, Napuli AJ, Creamer R, Dolja VV. Cell-to-cell movement and assembly of a plant closterovirus: roles for the capsid proteins and Hsp70 homolog. *EMBO J*. 2001;20:6997–7007. <https://doi.org/10.1093/emboj/20.24.6997>.
- Atabekova AK, Solovieva AD, Chergintsev DA, Solovyev AG, Morozov SY. Role of plant virus movement proteins in suppression of host RNAi defense. *Int J Mol Sci*. 2023;24:9049. <https://doi.org/10.3390/ijms24109049>.
- Cañizares MC, Lozano-Durán R, Canto T, Bejarano ER, Bisaro DM, Navas-Castillo J, et al. Effects of the crinivirus coat protein-interacting plant protein SAHH on post-transcriptional RNA silencing and its suppression. *Mol Plant Microbe Interact*. 2013;26:1004–15. <https://doi.org/10.1094/MPMI-02-13-0037-R>.
- Cao X, Zhao R, Wang H, Zhang H, Zhao X, Khan LU, et al. Genomic diversity of Areca Palm Velarivirus 1 (APV1) in Areca palm (*Areca catechu*) plantations in Hainan, China. *BMC Genomics*. 2021;22:725. <https://doi.org/10.1186/s12864-021-07976-6>.
- Carvalho SL, Tilsner J, Figueira AR, Carvalho CM. Subcellular localization and interactions among TGB proteins of Cowpea mild mottle virus. *Adv Virol*. 2022;167:2555–66. <https://doi.org/10.1007/s00705-022-05576-7>.
- Chen T, Wang HX, Meng R, Lan J, Xian SL, Feng XY, et al. Interaction of CP protein and CP protein of areca nut cryptosporidiosis virus 1 (ArPV1). *Mol Plant Breed*. 2024. <https://link.cnki.net/urlid/46.1068.s.20240326.1427.008>. (In Chinese). Epub 2024 Mar 28.
- Dao TNM, Kang SH, Bak A, Folimonova SY. A Non-conserved p33 protein of *Citrus tristeza* virus interacts with multiple viral partners. *Mol Plant Microbe Interact*. 2020;33:859–70. <https://doi.org/10.1094/MPMI-11-19-0328-FI>.
- Dashprakash M, Venkatesan G, Kumar A, Sankar M, Arya S, Ramakrishnan MA, et al. Prokaryotic expression, purification and evaluation of goatpox virus ORF117 protein as a diagnostic antigen in indirect ELISA to detect goatpox. *Adv Virol*. 2019;164:1049–58. <https://doi.org/10.1007/s00705-019-04170-8>.
- Fuchs M, Bar-Joseph M, Candresse T, Maree HJ, Martelli GP, Melzer MJ, et al. ICTV virus taxonomy profile: *Closteroviridae*. *J Gen Virol*. 2020;101:364–5.
- Gan SX, Yang L, Ren CX, Miu Q, Cheng ZH, Ji YH. Subcellular localization and pathogenicity of the coat protein of chlorotic yellowing virus in cucurbit. *Plant Prot*. 2024;50:240–5.
- Hainan Provincial Bureau of Statistics, Survey Office of National Bureau of Statistics in Hainan. Hainan Statistical Yearbook 2023. China Statistics Press; 2023. p. 230.
- He HF, Zhao CC, Zhu CQ, Yan WL, Yan MH, Zhang ZL, et al. Discovery of novel whitefly vector proteins that interact with a virus capsid component mediating virion retention and transmission. *Int J Biol Macromol*. 2023;226:1154–65. <https://doi.org/10.1016/j.jbiomac.2022.11.229>.
- Janik K, Schlink K. Unravelling the function of a bacterial effector from a non-cultivable plant pathogen using a yeast two-hybrid screen. *J vis Exp*. 2017;119: e55150. <https://doi.org/10.3791/55150>.
- Kokane SB, Kokane AD, Misra P, Warghane AJ, Kumar P, Gubayd MG, et al. In-silico characterization and RNA-binding protein based polyclonal antibodies production for detection of *Citrus tristeza* virus. *Mol Cell Probes*. 2020;54: 101654. <https://doi.org/10.1016/j.mcp.2020.101654>.
- Laimer J, Hofer H, Fritz M, Wegenkittl S, Lackner P. MAESTRO—multi agent stability prediction upon point mutations. *BMC Bioinform*. 2015;16:116. <https://doi.org/10.1186/s12859-015-0548-6>.
- Li J, Cao X, Jia X, Liu L, Cao H, Qin W, et al. Iron deficiency leads to chlorosis through impacting chlorophyll synthesis and nitrogen metabolism in *Areca catechu* L. *Front Plant Sci*. 2021;12: 710093. <https://doi.org/10.3389/fpls.2021.710093>.
- Liu Q, Zhang S, Mei S, Zhou Y, Wang J, Han GZ, et al. Viromics unveils extraordinary genetic diversity of the family *Closteroviridae* in wild citrus. *PLoS Pathog*. 2021;17: e1009751. <https://doi.org/10.1099/jgv.0.001397>.
- Mardanov ES, Ravin NV. Transient expression of recombinant proteins in plants using potato virus X based vectors. *Methods Enzymol*. 2021;660:205–22. <https://doi.org/10.1016/b.s.mie.2021.05.013>.
- Martelli GP. A brief historical account of the family *Closteroviridae*. *Methods Mol Biol*. 2019;2015:7–13. https://doi.org/10.1007/978-1-4939-9558-5_2.
- Miao R, Ye Z, MacFarlane S, Li Y, Mao Q, Tian Y, et al. The P3N-PIPO protein encoded by wheat yellow mosaic virus is a pathogenicity determinant and promotes its pathogenicity through interaction with NbRLK6 in *Nicotiana benthamiana*. *Viruses*. 2022;14:2171. <https://doi.org/10.3390/v14102171>.
- Mostert I, Bester R, Burger JT, Maree HJ. Identification of interactions between proteins encoded by Grapevine Leaf roll-Associated Virus 3. *Viruses*. 2023;15:208. <https://doi.org/10.3390/v15010208>.
- Paudel DB, Sanfaçon H. Exploring the diversity of mechanisms associated with plant tolerance to virus infection. *Front Plant Sci*. 2018;9:1575. <https://doi.org/10.3389/fpls.2018.01575>.
- Pires DE, Ascher DB, Blundell TL. DUET: a server for predicting effects of mutations on protein stability using an integrated computational approach. *Nucleic Acids Res*. 2014;42(Web server):W314–9. <https://doi.org/10.1093/nar/gku411>.
- Tan H, Zhao R, Wang H, Huang X. Identification and molecular characterization of a novel member of the genus *Totivirus* from *Areca catechu* L. *Adv Virol*. 2023;168:247. <https://doi.org/10.1007/s00705-023-05867-7>.
- Tatineni S, Gowda S, Dawson WO. Heterologous minor coat proteins of *Citrus tristeza* virus strains affect encapsidation, but the coexpression

- of HSP70h and p61 restores encapsidation to wild-type levels. *Virology*. 2010;402(2):262–70. <https://doi.org/10.1016/j.virol.2010.03.042>.
- Ur Rehman A, Li Z, Yang Z, Waqas M, Wang G, Xu W, et al. The coat protein of citrus yellow vein clearing virus interacts with viral movement proteins and serves as an RNA silencing suppressor. *Viruses*. 2019;11:329. <https://doi.org/10.3390/v11040329>.
- Wang X, Luo C, Xu Y, Zhang C, Bao M, Dou J, et al. Expression of the p24 silencing suppressor of Grapevine leafroll-associated virus 2 from Potato virus X or Barley stripe mosaic virus vector elicits hypersensitive responses in *Nicotiana benthamiana*. *Plant Physiol Biochem*. 2019;142:34–42. <https://doi.org/10.1016/j.plaphy.2019.06.033>.
- Wang H, Zhao R, Zhang H, Cao X, Li Z, Zhang Z, et al. Prevalence of Yellow Leaf Disease (YLD) and its associated Areca Palm Velarivirus 1 (APV1) in Betel palm (*Areca catechu*) plantations in Hainan, China. *Plant Dis*. 2020;104:2556–62. <https://doi.org/10.1094/PDIS-01-20-0140-RE>.
- Wee J, Wei GW. Benchmarking AlphaFold3's protein-protein complex accuracy and machine learning prediction reliability for binding free energy changes upon mutation. *ArXiv*:2406.03979v1. 2024.
- Wei Y, Shi Y, Han X, Chen S, Li H, Chen L, et al. Identification of cucurbit chlorotic yellows virus P4.9 as a possible movement protein. *Virol J*. 2019;16:82. <https://doi.org/10.1186/s12985-019-1192-y>.
- Xing ZY, Zhao RB, Cao XM, Wang HX. Screening of interaction proteins of coat protein 1 of areca yellow leaf virus. *Mol Plant Breed*. 2024;12:3912–21. **(In Chinese)**.
- Yang Z, Zhang Y, Wang G, Wen S, Wang Y, Li L, et al. The p23 of Citrus Tristeza Virus interacts with host FKBP-Type Peptidyl-Prolyl cis-Trans Isomerase 17–2 and is involved in the intracellular movement of the viral coat protein. *Cells*. 2021;10:934. <https://doi.org/10.3390/cells10040934>.
- Yang X, Luo X, Zhang Y, Zhang Z, OuYang X, Shi X, et al. Tomato chlorosis virus CPM protein is a pathogenicity determinant and suppresses host local RNA silencing induced by single-stranded RNA. *Front Microbiol*. 2023;14:1151747. <https://doi.org/10.3389/fmicb.2023.1151747>.
- Yu H, Qi S, Chang Z, Rong Q, Akinyemi IA, Wu Q. Complete genome sequence of a novel velarivirus infecting areca palm in China. *Adv Virol*. 2015;160:2367–70. <https://doi.org/10.1007/s00705-015-2489-9>.
- Zhao F, Maren NA, Kosentka PZ, Liao YY, Lu H, Dudit JR, et al. An optimized protocol for stepwise optimization of real-time RT-PCR analysis. *Hortic Res*. 2021;8(1):179. <https://doi.org/10.1038/s41438-021-00616-w>.
- Zhao S, Gong P, Ren Y, Liu H, Li H, Li F, et al. The novel C5 protein from tomato yellow leaf curl virus is a virulence factor and suppressor of gene silencing. *Stress Biol*. 2022;2:19. <https://doi.org/10.1007/s44154-022-00044-3>.