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Genetic variation and pathogenicity analysis of apple stem grooving virus-encoded coat protein

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Abstract

Apple stem grooving virus (ASGV) is one of the most significant pathogens infecting pome and stone fruit trees worldwide. The ASGV-encoded coat protein (CP) functions to encapsulate the viral genomic RNA and is essential for virus infection, while its genetic variation and pathogenicity remain largely unclear. Here, co-variation of the amino acids for ASGV CP and its implication have been analyzed. The results showed that, based on co-variation at amino acid positions 103 and 108, CP variants clustered into three groups, designated as types E¹⁰³-A¹⁰⁸, E¹⁰³-E¹⁰⁸, and Q¹⁰³-E¹⁰⁸. By using a potato virus X-based pathogenicity system, expression of CP (E¹⁰³-A¹⁰⁸)_{ASGV}, CP (E¹⁰³-E¹⁰⁸)_{ASGV}, and CP (Q¹⁰³-E¹⁰⁸)_{ASGV} led to attenuated disease symptoms and lower virus accumulation of potato virus X (PVX). Notably, type CP (Q¹⁰³-E¹⁰⁸)_{ASGV} exhibited lower virulence compared with the other two types. Transient expression assay in *Nicotiana benthamiana* plants demonstrated that the mutant type CP (Q¹⁰³-E¹⁰⁸)_{ASGV} accumulated in a significantly higher level than the other two types. Especially, in vivo and cell-free degradation assays revealed that the mutation of E¹⁰³ to Q¹⁰³ enhances protein stability probably via facilitating its escape from ubiquitin–26S proteasome pathway-mediated degradation. Furthermore, ASGV CP has been demonstrated to possess activity of viral suppressor for RNA silencing (VSR). Taken together, these results provide insights into understanding of the multiple roles in aspects of genetic variation, pathogenicity of heterologous PVX, protein homeostasis, and VSR activity of ASGV CP.

Keywords *Capillovirus*, Genetic variation, Protein stability, Ubiquitin–26S proteasome pathway, Potato virus X

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Background

Apple stem grooving virus (ASGV, *Capillovirus mali*) belongs to the subfamily *Trivirinae*, family *Betaflexiviridae* (Adams et al. 2016). The virions of ASGV are flexuous filament, 600–700 nm in length. The viral genome is composed of a positive-sense, single-stranded RNA with approximately 6500 nucleotides excluding the poly (A) tail at the 3' terminus (Yoshikawa et al. 1992). The genome contains two open reading frames (ORFs). ORF1 encodes a large replicase polyprotein (241 kDa) and a coat protein (CP) expressed from a sub-genomic RNA. ORF2 encodes a 36 kDa putative movement protein located within the ORF1 (Hirata et al. 2003; Tatineni et al. 2009). ASGV is recognized as one of the



most economically important viruses infecting pome and stone fruit trees around the world, and has a wide host range including apple, pear cultivars and species in the genera of *Prunus*, *Actinidia*, and *Citrus* (Ito et al. 2002; Clover et al. 2010; Li et al. 2022). ASGV infection in many commercial apple and pear cultivars are always latent in symptoms, but it can manifest symptoms like xylem grooving in trees grafted on *Malus pumila* cv. Virginia Crab (Lister 1970). ASGV is mainly transmitted by grafting, although its natural transmission vector remains unknown (Adams et al. 2016). Mixed infection of ASGV with other apple viruses including apple stem spitting virus (ASPV) and apple chlorotic leaf spot virus (ACLSV) frequently occurred in gardens (Hao et al. 2016).

The genomes of ASGV isolates exhibit significant nucleotide sequence variability. Several studies described the genomic variation and evolution of ASGV genomic sequences (Liebenberg et al. 2012; Chen et al. 2014a; Shokri et al. 2023). Two highly variable regions, variable region 1 from the coding regions of amino acids (aa) 532 to 570 and variable region 2 from aa 1583 to 1868, were identified in the ASGV genome. Phylogenetic analysis suggested that the diversity of ASGV genome was largely associated with host plant species (Liebenberg et al. 2012). Molecular variation analysis of ASGV full genomic sequences showed that the isolate ASGV-T47 shared 79.7%–97.6% nucleotide identity with other 15 isolates worldwide. Phylogenetic analysis indicated that ASGV-T47 and other 15 isolates clustered into two groups, while these isolates showed no correlation of host or geographical origin (Chen et al. 2014a). A recent study on evolution and biogeography of ASGV concluded that ASGV mostly likely originated and spread in East Asia, with a high nucleotide diversity observed in its Chinese population (Shokri et al. 2023).

As sub-genomic RNA-encoded viral CP protein is essential for virion formation, virus replication, and movement (Komatsu et al. 2012), molecular variation and evolution analysis of CP could provide information regarding virus genetic diversity and pathogenicity. Previously, studies on ACLSV CP showed that co-variation of five amino acids (aa) at positions 40, 59, 75, 130, and 184 were highly conserved within each cluster, and positions 40 and 75 were required for efficient infection (Yaegashi et al. 2007). A further study identified a new phylogenetic type (SHZ type), which was characterized by co-variation of amino acids at positions 40, 75, and 79 (Chen et al. 2014c). Even though the above findings identified several conserved residues of ACLSV CP and revealed the involvement of these residues in virus infection, the molecular analysis of co-variation and functional study on protein homeostasis of ASGV CP remain scarce.

In this study, the molecular variability and phylogenetic relationship of ASGV CP variants have been analyzed. Among which, three co-variation types including CP (E^{103} - A^{108}), CP (E^{103} - E^{108}), and CP (Q^{103} - E^{108}) were found based on variations at aa positions 103 and 108. By using a potato virus X (PVX)-based pathogenicity system, expression of these CP variants indicated that ASGV CP could attenuate PVX infection, which was associated with varied CP expression levels. In particular, the mutation of E^{103} to Q^{103} enhances protein stability, probably via preventing CP from ubiquitin–26S proteasome pathway-mediated degradation. Moreover, ASGV CP has been shown to possess activity of VSR. Altogether, our results provide insight into the genetic variations of ASGV CP and their influence on protein stability and PVX pathogenicity, facilitating our understanding on the multiple roles of ASGV CP.

Results

Co-variation analysis of amino acids at positions 103 and 108 in ASGV CP

During a survey of apple virus diseases in China, samples including leaves, branches, and fruits were collected and detected for ASGV by reverse-transcription PCR (RT-PCR) using specific primers (James 1999) targeting ASGV CP (Additional file 1: Table S1). Among the collected 60 samples, 45 were tested positive for ASGV (Additional file 1: Table S2). The full-length coding sequences of CP from these 45 samples were determined and translated into amino acid sequences, and then subjected to multiple sequence alignment along with 25 sequences retrieved from GenBank database (Additional file 1: Table S3 and S4) with ESPript 3.0 software online. The result revealed that the co-variation of two amino acids at positions 103 and 108 occurred among the total 70 isolates, which can be clustered into type I (E^{103} - E^{108} for 14 isolates), type II (E^{103} - A^{108} for 25 isolates), and type III (Q^{103} - E^{108} for 31 isolates) (Fig. 1a). A phylogenetic tree for CP sequences of these 70 isolates was conducted by neighbor-joining method with MEGA 11 based on protein Poisson distances, with branch support evaluated using 1000 bootstrap replicates (Tamura et al. 2021). Interestingly, these 70 isolates could also group into three clusters, and each corresponded fully to the three groups above based on the co-variation of aa at positions 103 and 108 (Fig. 1b).

To further investigate whether the co-variation at residues 103 and 108 of CP was conserved in the genus *Capillovirus*, several CP aa sequences belonging to other capilloviruses were selected and subjected to multiple sequence alignment. It was shown that, besides the E^{103} - E^{108} type for polycias capillovirus 1 isolate ZWY-16, no identical co-variation in other capilloviruses,

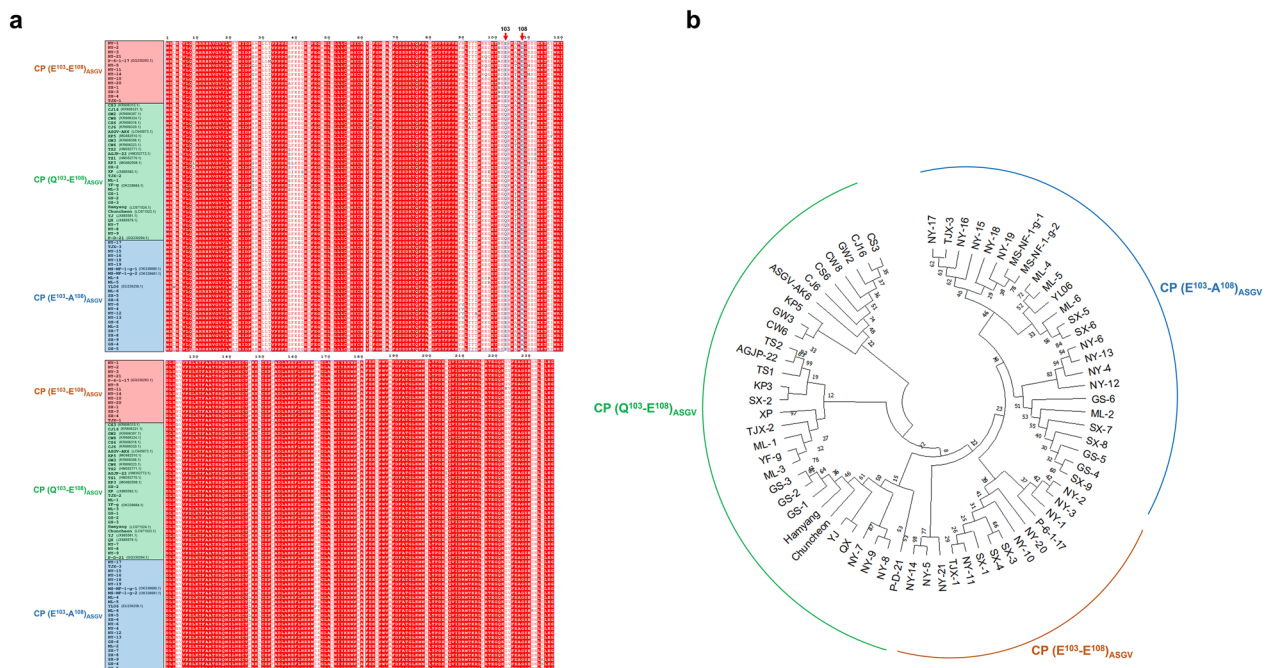


Fig. 1 Multiple sequence alignment and phylogenetic analysis of amino acid sequences of ASGV CP. **a** Alignment of CP sequences of ASGV isolates from GenBank (25 sequences) and obtained in this study (45 sequences) by ESPript 3.0 software online. Asterisks indicate two amino acids showing co-variation at positions 103 and 108. Three co-variation types $E^{103}-E^{108}$, $Q^{103}-E^{108}$, and $E^{103}-A^{108}$ were formed according to the co-variation at positions 103 and 108. **b** A phylogenetic tree of ASGV CP sequences from mentioned above. The tree was constructed by neighbor-joining method as implemented in MEGA 11 based on protein Poisson distance, with branch support evaluated by bootstrapping (1000 replicates). Three clusters (co-variation types $E^{103}-E^{108}$, $Q^{103}-E^{108}$, and $E^{103}-A^{108}$) were generated based on alignment in mentioned above

indicating the co-variation at positions 103 and 108 was not conserved in the genus *Capillovirus* (Additional file 2: Figure S1). These results suggested the co-variation of aa at positions 103 and 108 for ASGV CP.

ASGV CP attenuates the pathogenicity of PVX in *Nicotiana benthamiana* plants

To evaluate the role of CP for ASGV pathogenicity, we amplified the full-length coding region of CP ($E^{103}-A^{108}$)_{ASGV} fused with a Flag tag, and then inserted it into a PVX vector to generate PVX-CP ($E^{103}-A^{108}$)_{ASGV}-Flag (Fig. 2a). *Agrobacterium* containing PVX-CP ($E^{103}-A^{108}$)_{ASGV}-Flag was infiltrated into the leaves of *N. benthamiana* plants. Agroinfiltration of PVX and PVX-GFP served as controls. Symptom development was monitored on a daily basis. At 5 days post inoculation (dpi), obvious chlorosis and mosaic symptoms appeared on the upper leaves of PVX- and PVX-GFP-infected plants, while the PVX-CP ($E^{103}-A^{108}$)_{ASGV}-Flag-infected plants displayed only mild mosaic symptoms (Fig. 2b). To determine whether the attenuated symptoms on PVX-CP ($E^{103}-A^{108}$)_{ASGV}-Flag-infected plants were the consequence of reduced virus accumulation level, the upper

leaves were harvested for immunoblotting analysis with anti-PVX CP antibody. The result showed that the PVX viral multiplication level significantly decreased in PVX-CP ($E^{103}-A^{108}$)_{ASGV}-Flag-infected plants compared with the controls (Fig. 2c, d). The expression of CP ($E^{103}-A^{108}$)_{ASGV}-Flag and GFP was also determined (Fig. 2c). These results demonstrated that CP ($E^{103}-A^{108}$)_{ASGV} could weaken viral pathogenicity when expressed via a PVX vector.

The natural mutation at position 103 in ASGV CP affects the pathogenicity of PVX

The above phylogenetic and co-variation analysis of CP prompted us to examine whether the other two variants of CP, including CP ($E^{103}-E^{108}$)_{ASGV} and CP ($Q^{103}-E^{108}$)_{ASGV}, could also affect PVX pathogenicity. We separately introduced mutations of $E^{103}-E^{108}$ and $Q^{103}-E^{108}$ by amplifying with the template of CP ($E^{103}-A^{108}$)_{ASGV}, thus to ensure no change on other nucleotides. Flag-tagged CP mutants including CP ($E^{103}-E^{108}$) and CP ($Q^{103}-E^{108}$) were constructed into PVX vector to generate PVX-CP ($E^{103}-E^{108}$)_{ASGV}-Flag and PVX-CP ($Q^{103}-E^{108}$)_{ASGV}-Flag (Fig. 3a). The recombinant PVX-CP ($E^{103}-A^{108}$)_{ASGV}-Flag, PVX-CP ($E^{103}-E^{108}$)_{ASGV}-Flag, and PVX-CP ($Q^{103}-E^{108}$)_{ASGV}-Flag

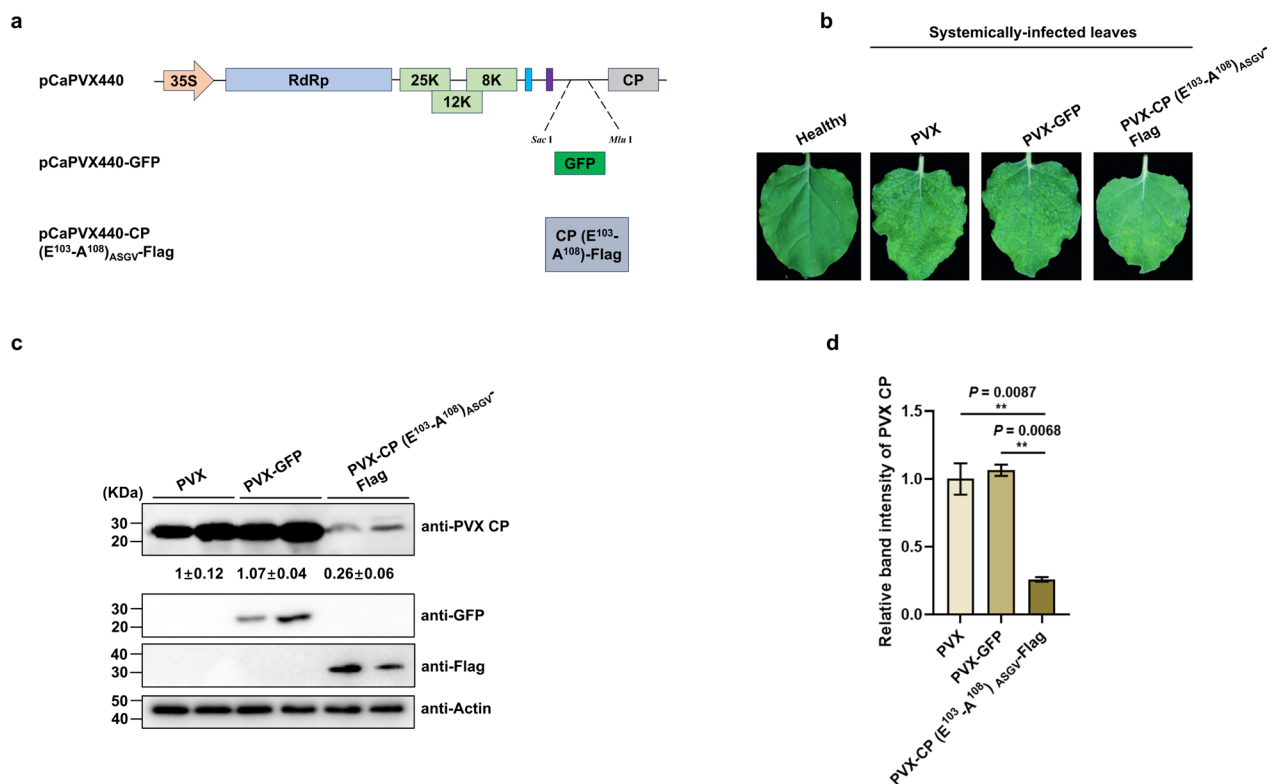


Fig. 2 ASGV CP reduces the pathogenicity of PVX in *N. benthamiana*. **a** Schematic representation of pCaPVX440 vector encoding various open reading frames (ORFs). The cyan and purple boxes indicate CP subgenomic promoters from PVX and tobacco mosaic virus (TMV), respectively. Coding sequence of GFP and Flag-tagged ASGV CP (E¹⁰³-A¹⁰⁸) were separately cloned into pCaPVX440 by restriction using enzymes *Sac* I and *Mlu* I to generate pCaPVX440-GFP and pCaPVX440-CP (E¹⁰³-A¹⁰⁸)_{ASGV}-Flag. **b** Symptom appearance on upper leaves of *N. benthamiana* plants inoculated with PVX, PVX-GFP, and PVX440-CP (E¹⁰³-A¹⁰⁸)_{ASGV}-Flag. Viral symptoms on systemically infected leaves were photographed at 5 days post inoculation (dpi). **c** Immunoblotting analysis of PVX CP accumulation levels. Band intensity of PVX CP was quantified using ImageJ. Expression levels of GFP and CP (E¹⁰³-A¹⁰⁸)_{ASGV}-Flag was determined. Actin served as loading control. **d** Band intensity of PVX CP in **c** was quantified and presented by histogram. **, $P < 0.01$

vectors were individually agroinfiltrated into *N. benthamiana* leaves. Agroinfiltration of PVX and PVX-GFP served as controls. At 5 dpi, severe viral symptoms appeared in the systemically infected leaves of PVX- and PVX-GFP-infected plants (Fig. 3b). In contrast, all the three PVX-CP_{ASGV} types caused milder disease symptoms (Fig. 3b). Interestingly, we found that the viral symptoms on PVX-CP (E¹⁰³-A¹⁰⁸)_{ASGV}-Flag-infected plants were comparable to that of the PVX-CP (E¹⁰³-E¹⁰⁸)_{ASGV}-Flag, whereas PVX-CP (Q¹⁰³-E¹⁰⁸)_{ASGV}-Flag-infected plants exhibited the mildest symptoms (Fig. 3b). Reverse transcription-quantitative PCR (RT-qPCR) analysis suggested that the accumulation levels of PVX genomic RNA in PVX-CP (Q¹⁰³-E¹⁰⁸)_{ASGV}-Flag-infected plants was notably lower than that of the other seedlings (Fig. 3c). Consistently, immunoblotting analysis showed that the accumulation level in PVX CP in PVX-CP (Q¹⁰³-E¹⁰⁸)_{ASGV}-Flag-infected plants accumulates with the least amount than other controls (Fig. 3d, e). These

findings suggest that ectopic expression of ASGV CP could reduce pathogenicity of PVX, and the pathogenicity was distinguished by variation at the conserved position 103.

The three co-variation types of ASGV CP localize in both cytoplasm and nucleus

Subcellular localization of viral proteins is crucial to determine their putative roles during virus infection. To examine the subcellular localization of the above ASGV CP variants, full-length coding sequences of CP (E¹⁰³-A¹⁰⁸)_{ASGV}, CP (E¹⁰³-E¹⁰⁸)_{ASGV}, and CP (Q¹⁰³-E¹⁰⁸)_{ASGV} were individually fused to the N-terminus of EGFP in pGD-EGFP vector to generate pGD-CP (E¹⁰³-A¹⁰⁸)_{ASGV}-EGFP, pGD-CP (E¹⁰³-E¹⁰⁸)_{ASGV}-EGFP, and pGD-CP (Q¹⁰³-E¹⁰⁸)_{ASGV}-EGFP (Fig. 4a). The corresponding constructs were agroinfiltrated in leaves of *N. benthamiana*. Confocal analysis showed that CP (E¹⁰³-A¹⁰⁸)_{ASGV}-EGFP, CP (E¹⁰³-E¹⁰⁸)_{ASGV}-EGFP, and CP

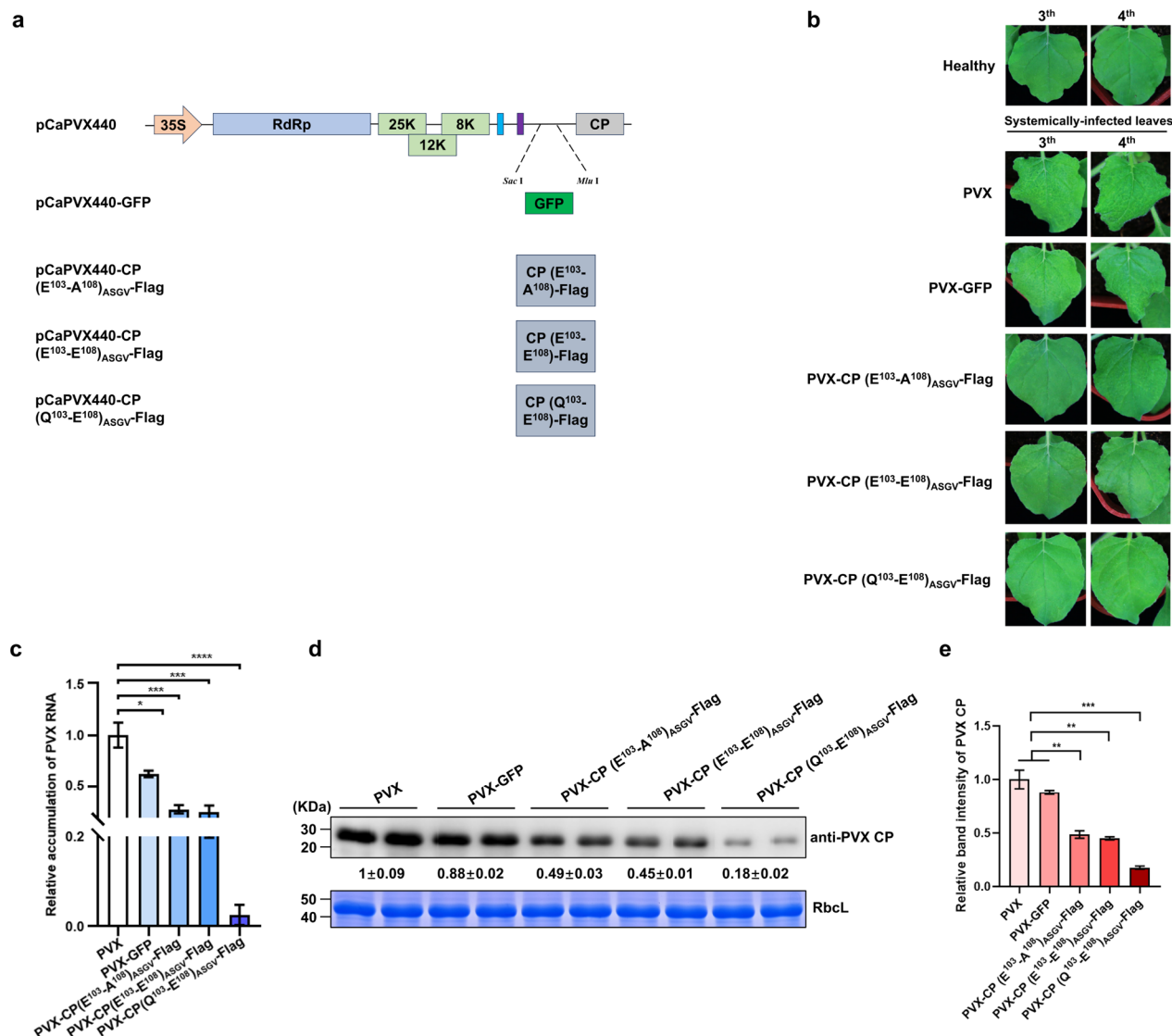


Fig. 3 The amino acid at position 103 determines the effect of ASGV CP on PVX pathogenicity. **a** Schematic representation of pCaPVX440 vector encoding various open reading frames (ORFs). Coding sequences of GFP and Flag-tagged ASGV CP variants were separately cloned into pCaPVX440 to generate pCaPVX440-GFP, pCaPVX440-CP (E¹⁰³-A¹⁰⁸)_{ASGV}-Flag, pCaPVX440-CP (E¹⁰³-E¹⁰⁸)_{ASGV}-Flag, and pCaPVX440-CP (Q¹⁰³-E¹⁰⁸)_{ASGV}-Flag. **b** Viral symptoms on upper leaves of *N. benthamiana* plants inoculated with PVX, PVX-GFP, PVX440-CP (E¹⁰³-A¹⁰⁸)_{ASGV}-Flag, pCaPVX440-CP (E¹⁰³-E¹⁰⁸)_{ASGV}-Flag, and pCaPVX440-CP (Q¹⁰³-E¹⁰⁸)_{ASGV}-Flag at 5 dpi. **c** RT-qPCR analysis of viral RNA accumulation levels in upper leaves of *N. benthamiana* plants infected with different PVX recombinant vectors at 5 dpi. Asterisks indicate statistically significant difference based on two-tailed Student's t-test (*, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$). **d** Detection of PVX CP accumulation level in upper leaves of *N. benthamiana* plants infected with different PVX recombinant vectors. The band intensity of PVX CP was quantified by ImageJ. Rubisco large subunit (RbCL) served as loading control. **e** Statistical analysis of band intensity for PVX CP in **d**. **, $P < 0.01$; ***, $P < 0.001$

(Q¹⁰³-E¹⁰⁸)_{ASGV}-EGFP localized in both the nucleus and cytoplasm (Fig. 4b).

The mutation of E¹⁰³ to Q¹⁰³ enhances protein accumulation level of ASGV CP in *N. benthamiana* plants

During the observation of the subcellular localization of three CP_{ASGV} variants, we found that the fluorescent intensity emitted by CP (Q¹⁰³-E¹⁰⁸)_{ASGV}-EGFP was

higher than that of the CP (E¹⁰³-A¹⁰⁸)_{ASGV}-EGFP and CP (E¹⁰³-E¹⁰⁸)_{ASGV}-EGFP (Fig. 3b). This observation prompted us to investigate whether CP (Q¹⁰³-E¹⁰⁸)_{ASGV} accumulates at a higher protein level than the other two CP variants. To determine the protein accumulation levels of these three CP variants, total protein from the infiltrated leaves of *N. benthamiana* plants transiently expressing CP (E¹⁰³-A¹⁰⁸)_{ASGV}-EGFP, CP

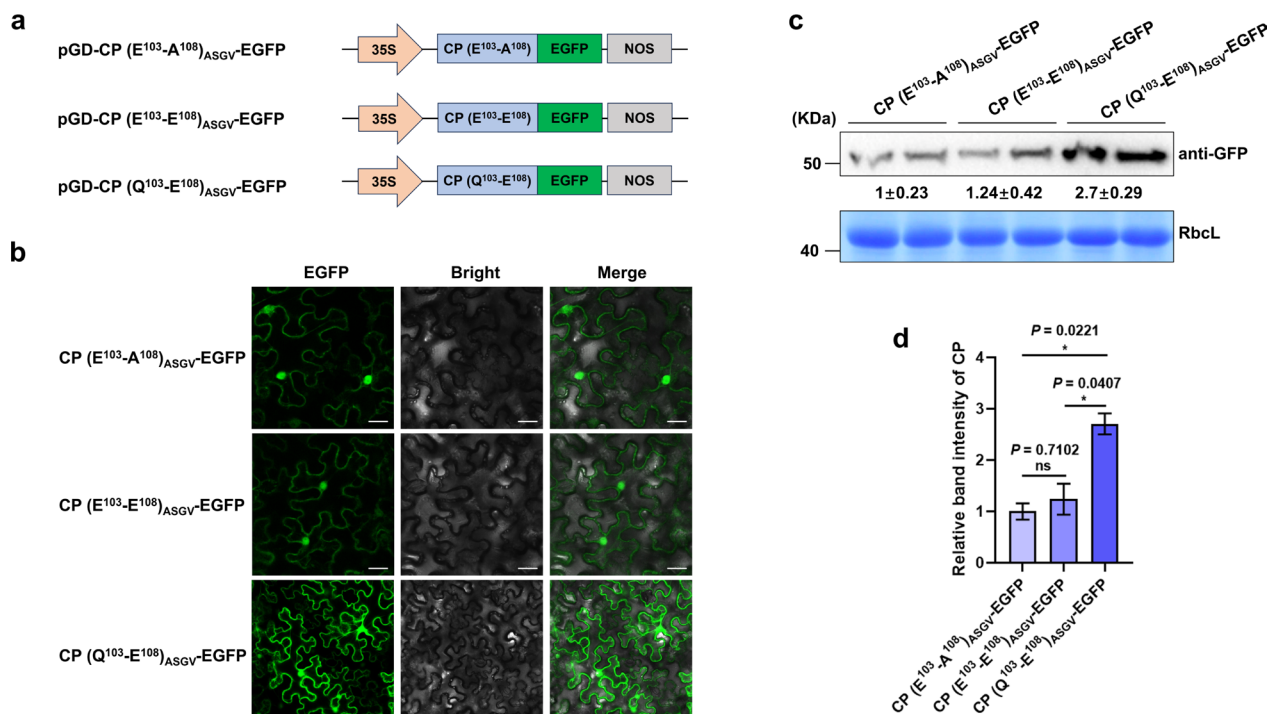


Fig. 4 Analysis of subcellular localization and protein expression levels of ASGV CP variants. **a** Schematic representation of recombinant constructs pGD-CP (E¹⁰³-A¹⁰⁸)_{ASGV}, pGD-CP (E¹⁰³-E¹⁰⁸)_{ASGV}, and pGD-CP (Q¹⁰³-E¹⁰⁸)_{ASGV}. CP (E¹⁰³-A¹⁰⁸)_{ASGV}, CP (E¹⁰³-E¹⁰⁸)_{ASGV}, and CP (Q¹⁰³-E¹⁰⁸)_{ASGV} were amplified and cloned into pGD-EGFP vector to generate corresponding constructs. **b** Subcellular localization of ASGV CP variants. EGFP-tagged CP (E¹⁰³-A¹⁰⁸)_{ASGV}, CP (E¹⁰³-E¹⁰⁸)_{ASGV}, and CP (Q¹⁰³-E¹⁰⁸)_{ASGV} were transiently expressed in *N. benthamiana* leaves, and the infiltrated regions were excised and analyzed by confocal microscope at 3 dpi. Scale bar = 25 μm. **c** Immunoblotting to determine the accumulation level of CP (E¹⁰³-A¹⁰⁸)_{ASGV}-EGFP, CP (E¹⁰³-E¹⁰⁸)_{ASGV}-EGFP, and CP (Q¹⁰³-E¹⁰⁸)_{ASGV}-EGFP. Total protein was extracted from *N. benthamiana* leaves in **b**, and subjected to SDS-PAGE and probed with anti-GFP antibody. The band intensity of EGFP-fused proteins was quantified with ImageJ. RbcL was used as loading control. **d** Statistical analysis of band intensity of CP (E¹⁰³-A¹⁰⁸)_{ASGV}-EGFP, CP (E¹⁰³-E¹⁰⁸)_{ASGV}-EGFP, and CP (Q¹⁰³-E¹⁰⁸)_{ASGV}-EGFP in **c**. *, $P < 0.05$; ns, no significant difference

(E¹⁰³-E¹⁰⁸)_{ASGV}-EGFP, or CP (Q¹⁰³-E¹⁰⁸)_{ASGV}-EGFP was analyzed by immunoblotting. The result showed that CP (Q¹⁰³-E¹⁰⁸)_{ASGV}-EGFP protein accumulated at a significantly higher level than either CP (E¹⁰³-A¹⁰⁸)_{ASGV}-EGFP or CP (E¹⁰³-E¹⁰⁸)_{ASGV}-EGFP, while it is comparable for protein amount between CP (E¹⁰³-A¹⁰⁸)_{ASGV}-EGFP and CP (E¹⁰³-E¹⁰⁸)_{ASGV}-EGFP (Fig. 4c, d). The results above indicated that variation of E to Q at position 103 improved protein accumulation level of ASGV CP.

The E¹⁰³ to Q¹⁰³ mutation attenuates degradation of ASGV CP by 26S proteasome pathway

We next investigated the underlying mechanism regulating the higher accumulation level of CP (Q¹⁰³-E¹⁰⁸) than the other two variants, CP (E¹⁰³-A¹⁰⁸) and CP (E¹⁰³-E¹⁰⁸). Considering that protein degradation is a common strategy to regulate protein homeostasis (Vu et al. 2018), we wondered whether CP would be degraded. For this, CP (E¹⁰³-A¹⁰⁸)-EGFP and EGFP control were separately expressed in *N. benthamiana* leaves via agroinfiltration. The 100 μM of translation inhibitor cycloheximide

(CHX) was treated for 4 h before harvesting for immunoblotting analysis. The results showed that CHX treatment evidently decreased the accumulation of CP (E¹⁰³-A¹⁰⁸)-EGFP, while no clear change was observed for EGFP control (Additional file 2: Figure S2), indicating the degradation of ASGV CP in vivo.

To examine whether ASGV CP was degraded by ubiquitin-26S proteasome or autophagy pathways, CP (E¹⁰³-A¹⁰⁸)-EGFP was transiently expressed in *N. benthamiana* leaves, followed by treatment with 100 μM of MG132 (an inhibitor of ubiquitin-26S proteasome pathway) or imethylsulfoxide (DMSO), and 3-MA (an inhibitor of autophagy pathway) or H₂O, which were supplemented with CHX. Immunoblotting results indicated that CP (E¹⁰³-A¹⁰⁸)-EGFP accumulated at a higher amount by the treatment with MG132 but not the 3-MA (Fig. 5a, Additional file 2: Figure S3), variants of CP (E¹⁰³-E¹⁰⁸)-EGFP and CP (Q¹⁰³-E¹⁰⁸)-EGFP showed similar results (Fig. 5b, c). To determine the in vivo ubiquitination of CP, the three CP variants were expressed in *N. benthamiana* leaves, followed by

immunoprecipitation with GFP beads and detected with anti-ubiquitin antibody. The results revealed the ubiquitination of CP variants in vivo (Fig. 5d). These results suggested that ASGV CP was degraded via ubiquitin–26S proteasome pathway but not the autophagy pathway.

The ubiquitin–26S proteasome pathway-mediated ASGV CP degradation prompted us to determine whether the higher protein level of CP (Q¹⁰³-E¹⁰⁸)-EGFP than the other two variants was attributed to its less

degradation. For this purpose, a cell-free degradation assay was performed. The leaf extracts from *N. benthamiana* expressing CP (E¹⁰³-A¹⁰⁸)-EGFP, CP (E¹⁰³-E¹⁰⁸)-EGFP, or CP (Q¹⁰³-E¹⁰⁸)-EGFP was incubated at 25°C with addition of 100 μM CHX and 1 mM ATP. Equal amounts of the corresponding extracts were taken at 0-, 30-, 60-, and 90-min post incubation, and subjected to immunoblotting analysis. The result showed that CP (Q¹⁰³-E¹⁰⁸)-EGFP degradation was slower than that of the CP (E¹⁰³-A¹⁰⁸)-EGFP and CP (E¹⁰³-E¹⁰⁸)-EGFP

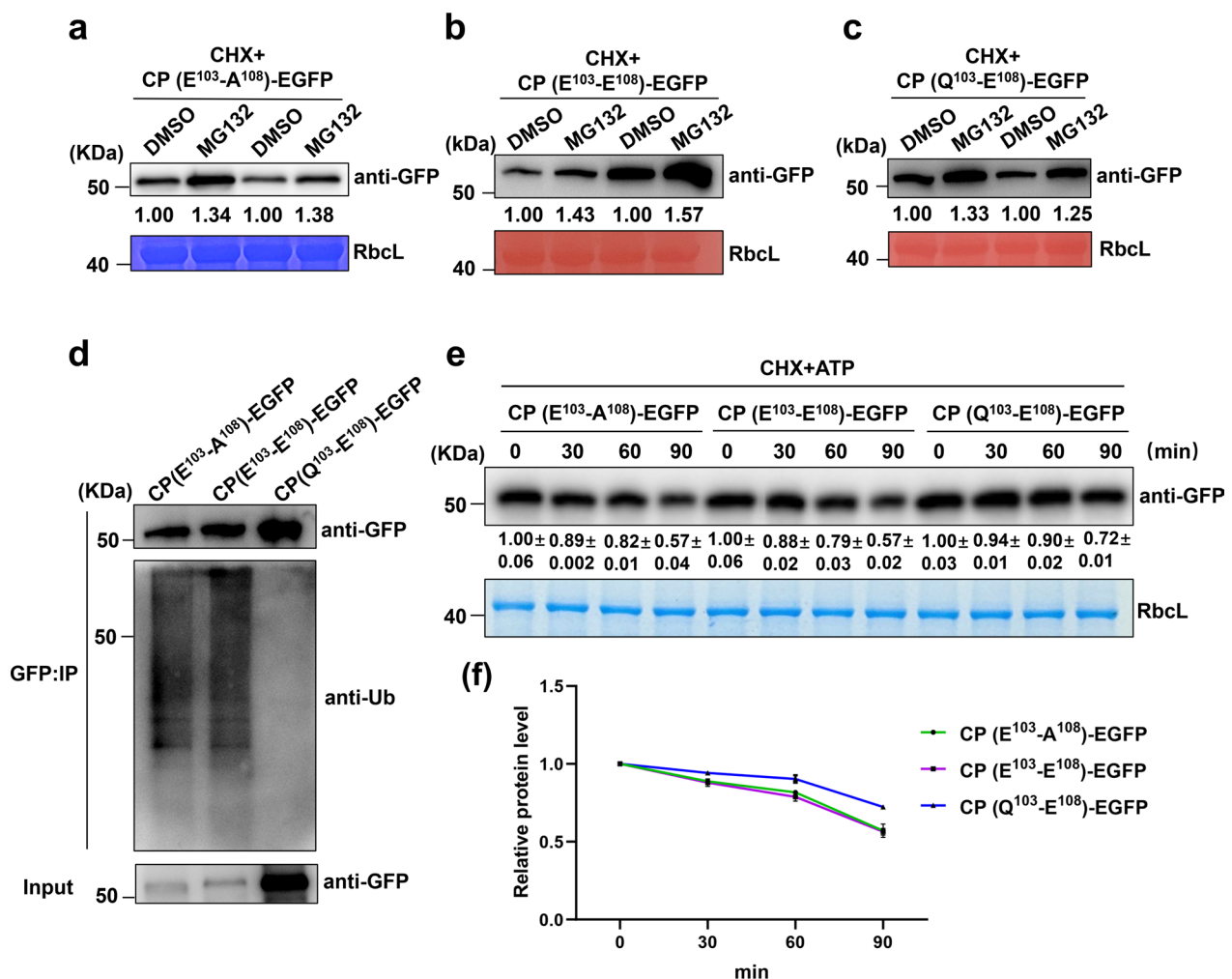


Fig. 5 Mutation from E¹⁰⁸ to Q¹⁰³ stabilizes protein accumulation level of ASGV CP accompanied by the decrease of ubiquitination level. **a-c** Three CP variants encompassing CP (E¹⁰³-A¹⁰⁸)-GFP, CP (E¹⁰³-E¹⁰⁸)-GFP, and CP (Q¹⁰³-E¹⁰⁸)-GFP were degraded via 26S proteasome pathway. The CP variants were separately expressed in *N. benthamiana* leaves, and then treated with 100 μM MG132 or an equal concentration of DMSO for 16 h before harvesting for immunoblotting analysis. RbcL served as loading control. **d** In vivo ubiquitination analysis of CP variants by immunoblotting. Total proteins from *N. benthamiana* leaves transiently expressing CP (E¹⁰³-A¹⁰⁸)-GFP, CP (E¹⁰³-E¹⁰⁸)-GFP, or CP (Q¹⁰³-E¹⁰⁸)-GFP were extracted and immunoprecipitated with anti-GFP agarose beads. The immunoprecipitated products were probed with anti-ubiquitin and anti-GFP antibodies. **e** Analysis of stability of ASGV CP via cell-free degradation assays. The *N. benthamiana* plants were infiltrated with *Agrobacterium* harboring CP (E¹⁰³-A¹⁰⁸)-GFP, CP (E¹⁰³-E¹⁰⁸)-GFP, and CP (Q¹⁰³-E¹⁰⁸)-GFP. At 3 dpi, leaf samples were collected. Then the leaf extracts were added with 1 mM ATP and 100 μM CHX, and incubated at 25 °C for indicated period of time. Immunoblotting analysis with anti-GFP antibody was performed to determine protein levels of CP. RbcL served as loading control. **f** A quantitative analysis of degradation rates of three variants of CP including CP (E¹⁰³-A¹⁰⁸)-GFP, CP (E¹⁰³-E¹⁰⁸)-GFP, and CP(Q¹⁰³-E¹⁰⁸)-GFP shown in **d**

(Fig. 5e, f). Consistently, the ubiquitination level of CP (Q^{103} -E ^{108})-EGFP was dramatically lower than the other two CP variants (Fig. 5d). These findings indicated that the lower ubiquitination level-mediated lower degradation rate of CP (Q^{103} -E ^{108}) contributes to the enhanced protein stability. Overall, these results suggested that ASGV CP is degraded via the ubiquitin–26S proteasome pathway, and the E ^{103} to Q ^{103} mutation enhances its protein stability via facilitating its escape from ubiquitin–26S proteasome pathway-mediated degradation.

ASGV CP shows activity for suppressor of gene silencing

To investigate whether ASGV CP functions as a VSR, the soluble modified GFP (smGFP) was co-expressed with CP (E ^{103} -A ^{108})-Flag, EV (pGD-3×Flag, as a negative control) or P19 (a vector expressing tomato bushy stunt virus p19 gene, as a positive control) by *Agrobacterium*-mediated expression assay in wide-type (WT) *N. benthamiana* plants. At 3 dpi, the intensity of green fluorescence in infiltrated regions was monitored under long-wavelength ultraviolet (UV) light. The intensity of green fluorescence in leaves expressing EV and smGFP decreased substantially (Fig. 6a). In contrast, leaf regions expressing P19 and smGFP showed strong intensity of fluorescence (Fig. 6a). Interestingly, we found that co-expression of smGFP with CP (E ^{103} -A ^{108})-Flag enhanced intensity of green fluorescence, which was higher than that of the negative control (Fig. 6a). Quantification of smGFP mRNA level revealed that the transcript level of smGFP in smGFP+CP (E ^{103} -A ^{108})-Flag was significantly higher than that of the smGFP+EV (Fig. 6b). Moreover, the accumulation levels of smGFP protein and expression of CP (E ^{103} -A ^{108})-Flag were analyzed by immunoblotting, showing that GFP amount was higher in leaves expressing smGFP+CP (E ^{103} -A ^{108})-Flag than that of the negative control (Fig. 6c), which was in agreement with the appearance of green fluorescence in infiltrated regions. Moreover, similar results were shown in GFP-transgenic 16c *N. benthamiana* plants (Fig. 6d–f). These results suggested that ASGV CP functions as a VSR to suppress post-transcriptional gene silencing (PTGS) in plants.

ASGV CP cannot elicit plant immune response

The results above suggest that expression of ASGV CP decreases PVX pathogenicity, we speculate that whether ASGV CP induces plant immune response. For this purpose, *Agrobacterium* containing pGD-EGFP or pGD-CP (E ^{103} -A ^{108})_{ASGV}-EGFP (without P19 expression) was infiltrated into the leaves of *N. benthamiana*. At 30 hpi, the infiltrated region of leaves was harvested for total RNA isolation and RT-qPCR analysis. The result showed that the expression levels of defense-related genes *NbPRI* and *NbWRKY40* (Qi et al. 2018) were not induced by

CP (E ^{103} -A ^{108})_{ASGV} (Additional file 2: Figure S4a, b). We also quantified the expression levels of *calmodulin-binding transcription activator-3* (*CAMTA3*), which regulates plant hormone salicylic acid-mediated immunity and antiviral RNAi response (Du et al. 2009; Wang et al. 2021). Similarly, CP (E ^{103} -A ^{108})_{ASGV} failed to alter expression level of *NbCAMTA3* (Additional file 2: Figure S4c). These results indicated that ASGV CP might be unable to induce plant defense response to restrict PVX infection.

Discussion

In this study, genetic variation and phylogenetic analysis of ASGV CP variants suggested that three types, including E ^{103} -A ^{108} , E ^{103} -E ^{108} , and Q ^{103} -E ^{108} , based on amino acids co-variation at positions 103 and 108. We then found that all three types of ASGV CP could reduce the pathogenicity of PVX. Interestingly, the different impacts of ASGV CP on pathogenicity of PVX were positively correlated with their co-variation-associated protein levels. Moreover, the E ^{103} to Q ^{103} mutation delayed the degradation of CP by ubiquitin–26S proteasome pathway, thus improving its protein stability. Finally, ASGV CP was characterized to possess VSR activity. Collectively, our studies comprehensively revealed the genetic variations of ASGV CP and their implication on protein homeostasis and pathogenicity of heterologous virus. These findings contributed to our understanding of the multifunctionality of ASGV CP.

PVX-based vectors have been extensively used to investigate the functions of proteins, including virus-encoded proteins. Several viral proteins were characterized as virulence factors or symptom determinants by using PVX vectors (Zhan et al. 2018; Li et al. 2020, 2021; Qiao et al. 2023). For instance, V2 and C1 proteins of apple geminivirus (AGV) were shown to enhance the pathogenicity of PVX, leading to severe crinkling and necrosis (Zhan et al. 2018). Ageratum leaf curl Sichuan virus (ALCScV)-encoded proteins C4 and C5 are pathogenicity determinants and enhance virus accumulation level of PVX (Li et al. 2020, 2021). A recent study illustrated that the P0 protein of a polerovirus causes severe symptoms in *N. benthamiana* plants when expressed via PVX vector (Qiao et al. 2023). Especially, ASPV CP was also demonstrated to be a pathogenetic determinant when expressed by PVX (Ma et al. 2019). However, different from those reported previously, here we found that ASGV CP attenuated the symptom and viral accumulation level of PVX. A previous study reported that ASGV infection downregulated or upregulated the expression of several defense/stress-related genes, and the authors speculated that the opposite expression pattern of these genes might lead to neutralized effect on plant growth and defense to induce asymptomatic infection (Chen et al. 2014b). As ASGV

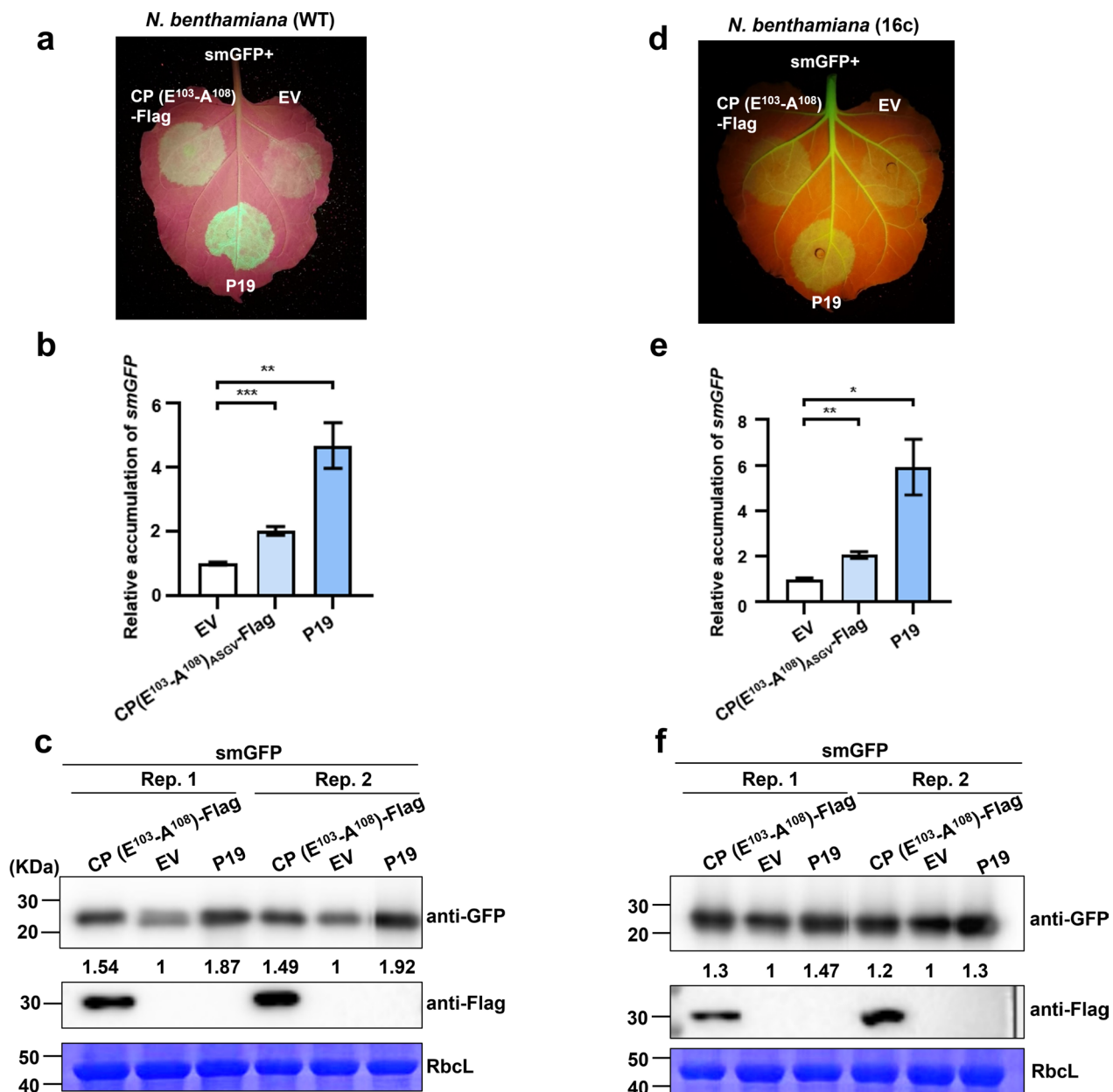


Fig. 6 Suppression of local RNA silencing by ASGV CP in both WT and 16c-transgenic *N. benthamiana* plants. **a, d** Green fluorescence shown in leaf regions agroinfiltrated with indicated combinations in WT and 16c-transgenic *N. benthamiana* plants under UV light at 3 dpi. **b, e** RT-qPCR to determine mRNA levels of *smGFP*. EV, CP (E¹⁰³-A¹⁰⁸)-Flag, and P19 were separately expressed in *N. benthamiana* leaves via agroinfiltration, total RNA was extracted and subjected to RT-qPCR analysis at 3 dpi. Asterisks indicate statistically significant difference based on two-tailed Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). **c, f** Immunoblotting analysis of protein accumulation levels of GFP and expression of CP (E¹⁰³-A¹⁰⁸)-Flag. The extracted proteins were probed with anti-GFP and anti-Flag antibodies. RbcL was used as a loading control. Band intensity was measured by ImageJ software and normalized to RbcL. Numbers below the top panel indicate the relative quantification of band intensity for each case. The molecular weight markers are shown on left of each panel

infection is usually symptomless in apple trees, ASGV CP might play roles in determining the asymptomatic infection via regulating apple gene expression.

Co-variation at specific residues of viral protein have been described for some apple viruses, and these

variations were shown to be associated with virus infection and phylogenetic clusters (Yaegashi et al. 2007; Chen et al. 2014c). In our attempt to examine the effect of co-variation types of ASGV CP on PVX pathogenicity, we found that expression of the variant CP (Q¹⁰³-E¹⁰⁸)

resulted in milder symptom and less virus accumulation than the variants CP (E^{103} -A ^{108}) and CP (E^{103} -E ^{108}). Moreover, transient expression assay suggested that CP (Q^{103} -E ^{108}) accumulates at a higher level than the other two co-variation types. These results indicated that the less pathogenetic PVX-CP (Q^{103} -E ^{108})_{ASGV} might be attributed to higher expression level of CP (Q^{103} -E ^{108}), hinting that for ASGV itself, the natural mutation of amino acid at position 103 from E to Q may increase protein stability of ASGV CP, thus facilitating ASGV virion assembly and virus replication. We wondered whether the co-variation at positions 103 and 108 was conserved for the genus *Capillovirus*. However, apart from the E^{103} -E ^{108} variation type for polycias capillovirus 1 isolate ZWY-16, the co-variation at positions 103 and 108 was not conserved in other species in the genus *Capillovirus*, implying the divergent evolution at residues 103 and 108 for species in this genus.

It was interesting that mutation of a single amino acid can substantially affect protein accumulation level. We thus suspect that CP might be degraded, while the mutation at position 103 from E to Q might be less targeted and degraded. As expected, in vivo ubiquitination and degradation assays demonstrated that CP was degraded via the ubiquitin–26S proteasome pathway. Further cell-free degradation results indicated that the mutation from E to Q at position 103 indeed attenuated the degradation of ASGV CP. Meanwhile, the ubiquitination level of CP (Q^{103} -E ^{108}) was notably lower than that of the other two CP variants. These findings revealed that, for ASGV CP, natural mutation of one residue at position 103 strikingly affects its ubiquitination level and protein stability.

Several lines of evidence suggested that the CPs encoded by some plant viruses function as VSR. For example, CPs encoded by cowpea mosaic virus, tomato ringspot virus, cocksfoot mottle virus, and pepino mosaic virus possess VSR activity (Cañizares et al. 2004; Karran and Sanfaçon 2014; Mathioudakis et al. 2014; Olspert et al. 2014). Notably, ASPV is a stone fruit trees-infecting virus, whose CP suppresses local RNA silencing (Ma et al. 2019). Similarly, by quantification of GFP intensity and mRNA level, we found that ASGV CP exhibits modest VSR activity. However, we failed to detect small interfering RNA of GFP by small RNA northern blotting for undetermined reasons.

Several viral proteins induce cell death in *N. benthamiana* leaves via PVX vector, usually accompanying with generation of reactive oxidative species (ROS) and induction of defense-related genes (Zhan et al. 2018; Ren et al. 2024). In this study, we showed that ASGV CP reduces pathogenicity and virus accumulation of PVX. An unresolved question is how ASGV CP attenuates PVX infection in *N. benthamiana*. Viral CPs are always recognized

by hosts and elicit host defense response (Callaway et al. 2001; Saito et al. 1989). Recently, rice stripe virus-encoded CP was shown to activate jasmonic acid signaling and anti-viral RNA silencing pathway (Yang et al. 2020; Huang et al. 2025). Our results suggested that ASGV CP cannot elicit immune response in *N. benthamiana*, as evidenced by inactivation of defense-related genes. A previous study suggested that AGV C1 protein potentially enhances the pathogenicity of PVX by increasing the accumulation of viral TGB sgRNA and CP sgRNA (Zhan et al. 2018). Therefore, we cannot rule out the probability that ASGV CP might function to reduce the amount of viral genomic RNA, thus attenuate the pathogenicity of PVX by an unknown mechanism. Alternatively, ASGV CP might interact with PVX-encoded protein(s) to disrupt their functions, leading to decreased PVX pathogenicity.

Conclusions

By analyzing the genetic variation and evolution of ASGV CP, we identified three co-variation types including CP (E^{103} -A ^{108}), CP (E^{103} -E ^{108}), and CP (Q^{103} -E ^{108}) based on variations at residue positions 103 and 108. The three CP variants attenuated the pathogenicity of PVX, which was associated with the dynamic levels of ASGV CP. Mutation of E^{103} to Q^{103} enhanced protein stability of CP probably via decreasing its ubiquitination level, thus contribute to its escape from ubiquitin–26S proteasome pathway-mediated degradation. Moreover, ASGV CP possesses VSR activity. These findings offer information and underlying mechanisms on the multifunctionality of the ASGV CP, providing multiple insights into the understanding of pathogenicity for ASGV and other related pome and stone fruit tree-infecting viruses.

Methods

Plants materials and growth conditions

The WT *N. benthamiana* and GFP-transgenic *N. benthamiana* line 16c were grown in a growth chamber set at 16 h light at 25 °C / 8 h dark at 23 °C photoperiod.

Plasmid construction

For transient expression assays in *N. benthamiana*, coding sequences of CP (E^{103} -A ^{108})_{ASGV}, CP (E^{103} -E ^{108})_{ASGV}, and CP (Q^{103} -E ^{108})_{ASGV} were cloned into the pGD-EGFP or pGD-3×Flag vectors (Xie et al. 2024).

For pathogenicity analysis of recombinant PVX vectors, three types of CP_{ASGV} variants were inserted into the pCaPVX440 expression vector. The empty pCaPVX440 and pCaPVX440-GFP served as controls (Wang et al. 2014). *Agrobacterium* containing these constructs were separately infiltrated into *N. benthamiana* leaves. Viral

symptom development and protein accumulation were monitored.

Sequence alignment and phylogenetic analysis

Amino acid sequences of the CP_{ASGV} gene from 70 isolates (45 from this study and 25 retrieved from GenBank) were analyzed. The 70 amino acid sequences were aligned using CLUSTALW in MEGA 11. A phylogenetic tree was constructed using the neighbour-joining (NJ) method with variance estimated using 1000 bootstrap replicates.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) analysis

Total RNA of *N. benthamiana* leaves was extracted using TRIzol (TransGen, Beijing, China) according to the manufacturer's instructions. cDNA synthesis was performed using 1 µg of total RNA and PC58-TRUEscript RT MasterMix (Aidlab, Beijing, China). The resulting cDNA served as a template for the subsequent qPCR assay with 2×ChamQ Blue Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). The *Nbactin* gene was used as the internal control. Relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Agrobacterium-mediated transient expression and immunoblotting analysis

Agrobacterium-mediated transient expression and immunoblotting assays were conducted as previously described (Cao et al. 2012; Xie et al. 2021). *Agrobacterium* containing respective plasmids were cultured at 28 °C overnight. The cultures were then centrifuged and resuspended in an infiltration buffer containing 10 mM MgCl₂, 10 mM MES, pH 5.7, and 100 µM acetosyringone. The corresponding cultures were incubated for 2–3 h before infiltration into 4-week-old *N. benthamiana* leaves.

For protein stability analysis, agroinfiltrated leaf regions were treated with 100 µM CHX for 4 h, or CHX plus 100 µM MG132 or 10 mM 3-methyladenine (3-MA), DMSO or H₂O for 16 h before harvesting for protein quantification.

Plant tissues were homogenized in extraction buffer (220 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM MgCl₂, 50 mM KCl) supplemented with 1% β-mercaptoethanol. The mixture was incubated on ice for 10 min, and then centrifuged at 10,000×g for 10 min at 4 °C. The supernatant obtained was heated at 100 °C for 5 min to denature the proteins. Then the protein extracts were separated by SDS-PAGE and probed with corresponding antibodies.

Cell-free degradation assay

Total proteins were extracted from *N. benthamiana* leaves expressing CP (E¹⁰³-A¹⁰⁸)-EGFP, CP (E¹⁰³-E¹⁰⁸)-EGFP, and CP (Q¹⁰³-E¹⁰⁸)-EGFP. The protein extracts were supplemented with 100 µM CHX and 1 mM ATP, and incubated at room temperature (25°C) for indicate periods of time. The protein samples were collected at indicated timepoints, and further subjected to immunoblotting analysis.

In vivo ubiquitination assay

In vivo ubiquitination assay was performed as previously described with minor modification (Chen et al. 2025). *Agrobacterium* containing CP (E¹⁰³-A¹⁰⁸)-EGFP, CP (E¹⁰³-E¹⁰⁸)-EGFP, or CP (Q¹⁰³-E¹⁰⁸)-EGFP were infiltrated into *N. benthamiana* leaves. At 3 dpi, total proteins were extracted from above samples using extraction buffer (220 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM MgCl₂, 50 mM KCl) supplemented with 1% β-mercaptoethanol. The protein extracts were incubated with GFP agarose beads at 4°C for 3 h, the beads washed three times with wash buffer (220 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM MgCl₂, 50 mM KCl). The immunoprecipitated products were then analyzed by immunoblotting using an anti-ubiquitin antibody (HUABIO, Hangzhou, China).

Confocal analysis

To determine the subcellular localization of three different variants of the CP protein, recombinant vectors expressing CP (E¹⁰³-A¹⁰⁸)-EGFP, CP (E¹⁰³-E¹⁰⁸)-EGFP, and CP (Q¹⁰³-E¹⁰⁸)-EGFP were constructed and transformed into *A. tumefaciens* strain GV3101. At 3 dpi, the infiltrated leaf regions were excised for analysis using confocal microscopy (Leica Microsystems, Wetzlar, Germany). For EGFP, the excitation wavelength was 488 nm, and the emission wavelength was detected between 500–550 nm.

Abbreviations

ACLSV	Apple chlorotic leaf spot virus
AGV	Apple geminivirus
ALCScV	Ageratum leaf curl Sichuan virus
ASGV	Apple stem grooving virus
ASPV	Apple stem spitting virus
ATP	Adenosinetriphosphate
CAMTA3	Calmodulin-binding transcription activator-3
CHX	Cycloheximide
CP	Coat protein
DMSO	Dimethylsulfoxide
Dpi	Days post inoculation
ORFs	Open reading frames
PTGS	Post-transcriptional gene silencing
PVX	Potato virus X
Rbcl	Rubisco large subunit
ROS	Reactive oxidative species
RT-qPCR	Reverse transcription-quantitative PCR

SmGFP Soluble modified GFP
UV Ultraviolet
VSR Viral suppressor for RNA silencing

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s42483-025-00407-4>.

Additional file 1: Figure S1. CP (E^{103} -A ^{108}) undergoes protein degradation. **Figure S2.** CP (E^{103} -A ^{108})-GFP is not degraded in an autophagy-dependent manner. **Figure S3.** ASGV CP is unable to induce plant defense response. **Figure S4.** ASGV CP is unable to induce plant defense response.

Additional file 2: Table S1. Primers for vectors construction. **Table S2.** Lists of geographic distribution and symptoms of apple samples tested positive for ASGV. **Table S3.** The detailed information of 25 ASGV CP sequences from GenBank database. **Table S4.** The 45 ASGV CP sequences cloned in this study. **Table S5.** Primers for qPCR analysis.

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Author contributions

JX, QY, ZZ, and TZ designed the experiments. JX and QY performed the experiments. JX, QY, HC, TY, LH, LZ, LS, SL, XY, ZF, ZZ, and TZ analyzed the data. JX, QY, and TZ wrote the manuscript. All authors approved the final version and publication of the manuscript.

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Data Availability

All data generated during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interests

The authors declare no competing interests.

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