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Interaction between cucumber green mottle mosaic virus MP and CP promotes virus systemic infection

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Abstract

The movement protein (MP) and coat protein (CP) of tobamoviruses play critical roles in viral cell-to-cell and long-distance movement, respectively. Cucumber green mottle mosaic virus (CGMMV) is a member of the genus *Tobamovirus*. The functions of CGMMV MP and CP during viral infection remain largely unclear. Here, we show that CGMMV MP can interact with CP in vivo, and the amino acids at positions 79–128 in MP are vital for the MP–CP interaction. To confirm this finding, we mutated five conserved residues within the residue 79–128 region and six other conserved residues flanking this region, followed by in vivo interaction assays. The results showed that the conserved threonine residue at the position 107 in MP (MP^{T107}) is important for the MP–CP interaction. Substitution of T107 with alanine (MP^{T107A}) delayed CGMMV systemic infection in *Nicotiana benthamiana* plants, but increased CGMMV local accumulation. Substitutions of another 10 conserved residues, not responsible for the MP–CP interaction, with alanine inhibited or abolished CGMMV systemic infection, suggesting that these 10 conserved residues are possibly required for the MP movement function through a CP-independent manner. Moreover, two movement function-associated point mutants (MP^{F17A} and MP^{D97A}) failed to cause systemic infection in plants without impacting on the MP–CP interaction. Furthermore, we have found that co-expression of CGMMV MP and CP increased CP accumulation independent of the interaction. MP and CP interaction inhibits the salicylic acid-associated defence response at an early infection stage. Taken together, we propose that the suppression of host antiviral defence through the MP–CP interaction facilitates virus systemic infection.

KEYWORDS
accumulation, coat protein, cucumber green mottle mosaic virus, interaction, movement protein

Ya-Juan Shi and Xue Yang contributed equally to this work.

1 | INTRODUCTION

Cucumber green mottle mosaic virus (CGMMV) was first found to cause disease in cucumber in England. Currently, this virus has been reported as a major threat to cucurbit production worldwide (Al-Shahwan & Abdalla, 1992; Antignus et al., 1990; Dombrovsky et al., 2017; Hilf & Dawson, 1993; Nagendran et al., 2015; Rajamony et al., 1990; Sugiyama et al., 2006; Wang & Chen, 1985).

Cucumber green mottle mosaic virus is a member of the genus *Tobamovirus*, family *Virgaviridae*. The genome of CGMMV is a single-stranded, positive-sense RNA of about 6.4 kb (Ugaki et al., 1991). CGMMV genomic RNA encodes four proteins, namely the 186, 129, 29, and 17.3 kDa proteins. Both 129 and 186 kDa proteins are known to participate in CGMMV replication and suppression of RNA silencing (Chen et al., 2020). The 29 and 17.3 kDa proteins are known as the movement protein and coat protein (MP and CP), respectively.

Tobamoviruses cause significant economic damage to crop production (Ishikawa et al., 2022; Ruiz et al., 2021; Sofy et al., 2021). The study of tobamovirus has greatly contributed to the development of plant virology (Fraile & García-Arenal, 2018). The MP of tobacco mosaic virus (TMV) is best-studied, and is required for TMV cell-to-cell movement and plasmodesmata (PD) targeting (Heinlein, 2015). In addition, TMV MP has been found to cause a stress-like response in plants (Dombrovsky et al., 2017). Apart from encapsidation of viral RNA, tobamovirus CPs are required for viral long-distance movement (Callaway et al., 2001) as well as suppression of salicylic acid (SA)-responsive gene expression (Conti et al., 2012; Rodriguez et al., 2014). Down-regulation of the SA signalling pathway by TMV CP is also required for virus systemic infection and stabilization of DELLA proteins to promote TMV systemic infection (Venturuzzi et al., 2021). Recent studies on CGMMV have found that the amino acid residues at positions 96 and 89 in the CP can affect virus infectivity (Liu et al., 2020; Zhang et al., 2018). TMV CP and MP interaction was observed in cells in the presence of N-terminus of yellow fluorescent protein and C-terminus of YFP (Bazzini et al., 2007). The CP of citrus yellow vein clearing virus interacts with all the three movement proteins (TGBp1, TGBp2, and TGBp3), and CP colocalizes with TGBp1 and TGBp3 at the PD of epidermal cells of *Nicotiana benthamiana* leaves (Ur Rehman et al., 2019). The MP of Sesbania mosaic virus (SeMV) interacts with its CP (Chowdhury & Savithri, 2011).

In contrast to the earlier reports on TMV MP and CP, studies on CGMMV MP and CP and their interaction during virus infection is very limited. In this study we have found that CGMMV MP and CP can interact with each other, and the key MP amino acid residues responsible for the interaction with CP have been determined. We have found that the movement function of MP is dispensable for the MP–CP interaction, and the MP–CP interaction promotes disease symptom development. Furthermore, we have also found that MP and CP co-expression can increase CP accumulation. The MP–CP interaction inhibits SA-associated defence response at early infection stage. We propose that the suppression of host antiviral defence through the MP–CP interaction facilitates CGMMV systemic infection.

2 | RESULTS

2.1 | Interaction between CGMMV MP and CP in vivo

The interaction between CGMMV P2 (the 57 kDa readthrough part between 129 and 186 kDa), MP, and CP was tested in *N. benthamiana* leaf cells using bimolecular fluorescence complementation (BiFC) assays. Agrobacteria harbouring constructs pMP-nYFP (N-terminus of YFP), pMP-cYFP (C-terminus of YFP), pCP-nYFP, pCP-cYFP, and pP2-cYFP were co-infiltrated, in various combinations, into *N. benthamiana* leaves. The combinations of pMP-nYFP and pP2-cYFP, pCP-nYFP and pP2-cYFP were used as negative controls. By 2 days post-agro-infiltration (dpai), strong YFP fluorescence was observed in the cells in *N. benthamiana* leaves co-infiltrated with pMP-nYFP and pCP-cYFP, pCP-cYFP and pCP-nYFP, or pCP-nYFP and pMP-cYFP. The strong YFP fluorescence appeared as punctate signals in different parts of the cytoplasm (Figure 1a). Furthermore, co-immunoprecipitation (Co-IP) assays indicated that MP interacted with CP in vivo (Figure 1b).

To further confirm the interaction between CGMMV MP and CP, we co-expressed YFP fused to the C-terminus of CP (CP-YFP) and red fluorescent protein [RFP] fused to the C-terminus of MP (MP-RFP) fusions (CP-YFP+MP-RFP) in cells of *N. benthamiana* leaves. As shown in Figure 2a,b, the MP-RFP was indeed colocalized with CP-YFP in the cytoplasm. The colocalization of CP-YFP and MP-RFP was further verified through coefficient analysis (Figure 2c) as previously reported (Zinchuk & Grossenbacher-Zinchuk, 2009). When MP-RFP or CP-YFP was expressed individually in *N. benthamiana* cells, the MP-RFP fluorescence appeared as punctate signals in the cytoplasm, while the CP-YFP fluorescence appeared as free signals in the cytoplasm and nucleus (Figure 2d,e).

2.2 | Amino acid residues 79–128 in the MP are responsible for the interaction with CP

To determine which domain in the MP is necessary for the interaction with CP, we first fused the MP N terminal 50 amino acid residues to the N terminus of YFP to produce $pMP_{1\text{-}50}$-nYFP and the amino acid residues 51–264 of the MP to the C terminus of YFP to produce $pMP_{51\text{-}264}$-cYFP, based on a report by Yuan et al. (2016). Plasmid combinations of $pMP_{1\text{-}50}$-nYFP/CP-cYFP and $pMP_{51\text{-}264}$-cYFP/CP-nYFP were co-expressed in *N. benthamiana* leaves (Figure 3a). Co-infiltration of $MP_{1\text{-}50}$-nYFP and CP-cYFP into *N. benthamiana* leaves yielded YFP fluorescence in cells by 2 dpi (Figure 3b). Co-expression of $MP_{51\text{-}264}$-cYFP and CP-nYFP in *N. benthamiana* leaf cells produced fluorescent punctate signals near the cell walls. Further analyses of the interactions between $MP_{51\text{-}182}$-cYFP and CP-nYFP, and $MP_{183\text{-}264}$-cYFP and CP-nYFP indicated that the residues 51–182 of the MP were responsible for formation of the fluorescent punctate signals near the cell walls (Figure 3b). Analysis of the MP amino acid sequence using MEME online software (https://meme-suite.org/meme/tools/meme) identified three motifs in the region encompassing amino acid residues 51–182 (Figure 3c). These three fragments were cloned into the p35S-nYFP

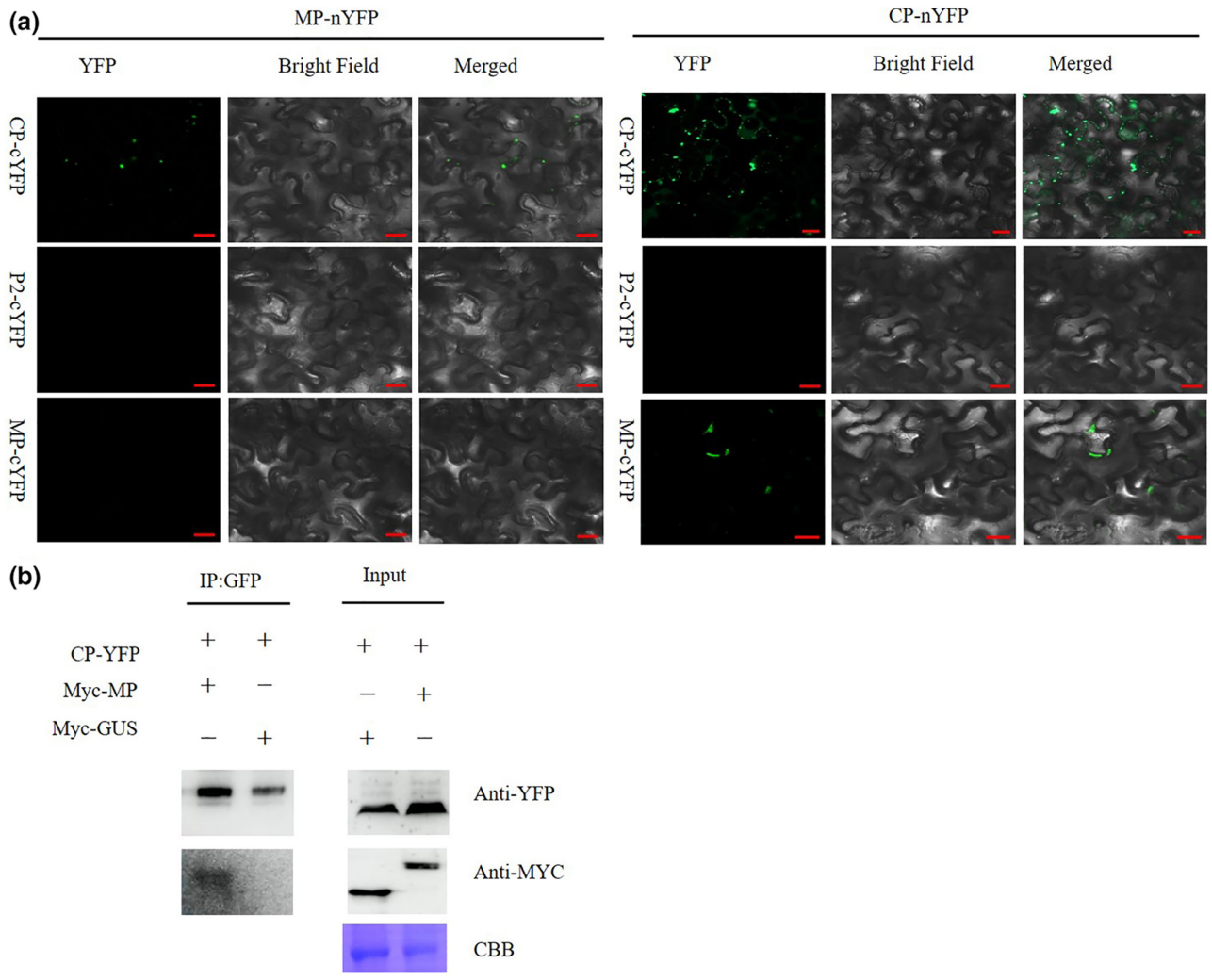
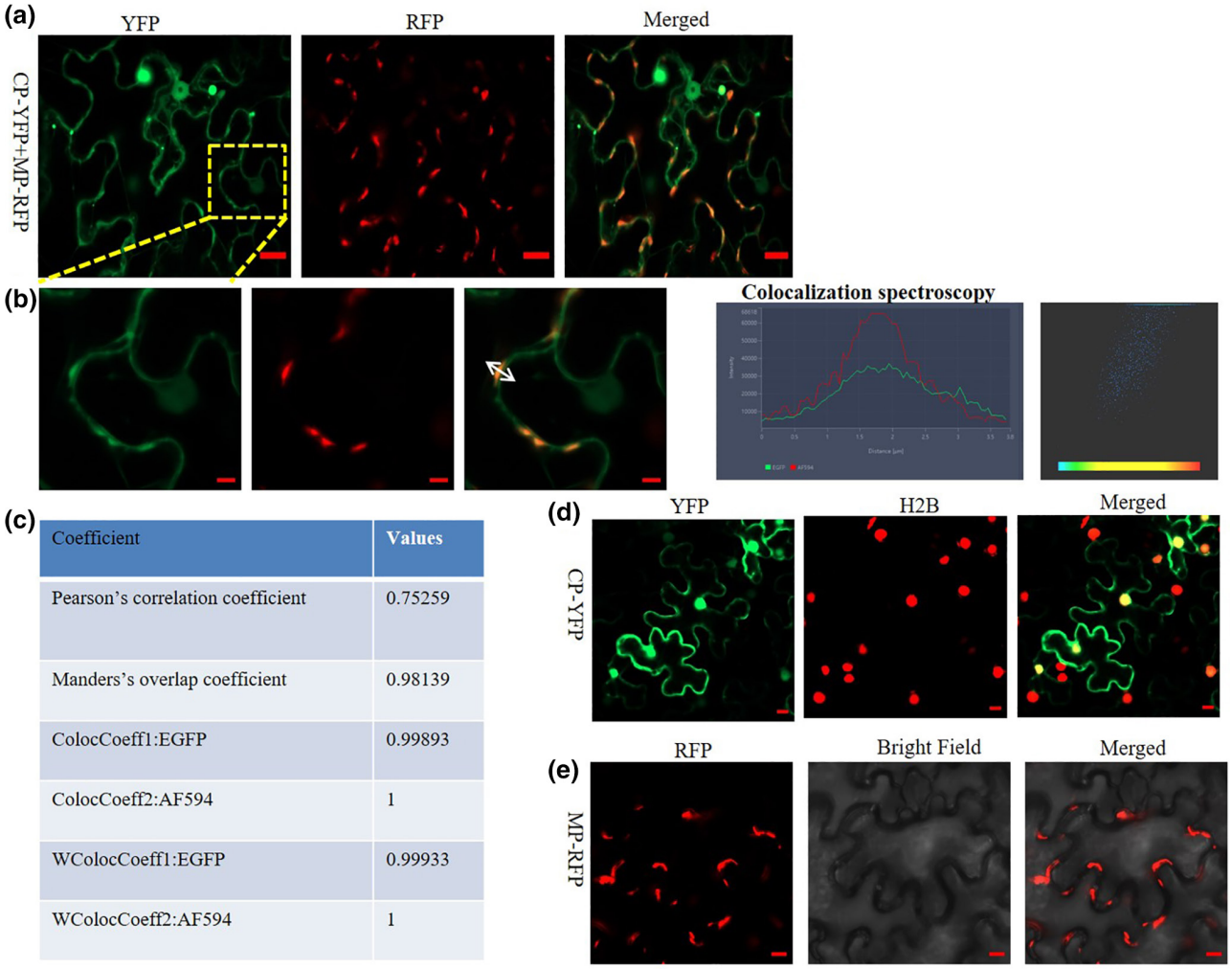


FIGURE 1 Cucumber green mottle mosaic virus (CGMMV) movement protein (MP) can interact with coat protein (CP) in vivo. (a) CGMMV MP and CP were fused to the N-terminus of YFP (MP-nYFP and CP-nYFP) and transiently co-expressed with CP-cYFP, P2-cYFP or MP-cYFP in *Nicotiana benthamiana* leaf cells. The infiltrated leaves were harvested at 2 days post-agro-infiltration (dpai), and examined and imaged under a LSM710 laser scanning microscope. Bar = 20 μm. (b) The interaction analysis of MP and CP in vivo by co-immunoprecipitation (Co-IP). *N. benthamiana* leaves were co-infiltrated with *Agrobacterium tumefaciens* cells harbouring expression vectors to express CP-YFP and Myc-MP, CP-YFP and Myc-GUS. Leaf protein extracts were incubated with GFP beads. Samples before (input) and after (IP) immunoprecipitation were analysed by immunoblotting using anti-GFP or anti-Myc antibody

vector to produce plasmids $pMP_{50\text{-}78}$-nYFP, $pMP_{79\text{-}128}$-nYFP, and $pMP_{130\text{-}163}$-nYFP, and were then co-expressed, individually, with CP-cYFP in *N. benthamiana* leaves through agro-infiltration. By 2 dpi, the leaves co-infiltrated with $pMP_{79\text{-}128}$-nYFP and pCP-cYFP produced fluorescent punctate signals near the cell walls, while the leaves co-infiltrated with $pMP_{50\text{-}78}$-nYFP and pCP-cYFP or $pMP_{130\text{-}163}$-nYFP and pCP-cYFP did not (Figure 3d), indicating that the motif encompassing residues 79–128 is crucial for the interaction between MP and CP.

2.3 | Residue T107 in the MP is important for the interaction with CP

To determine the key amino acid in the MP that is necessary for the interaction with CP, we performed an alignment using the amino acid sequence of CGMMV MP and that of other tobamovirus MPs with the online PROMALS3D software (http://prodata.swmed.edu/promals3d/promals3d.php) (Figure S1). Five conserved amino acids (C87, R99, E105, T107, and Y128) in the MPs were found to harbour hydroxyl groups and were substituted with alanine. The mutated sequences were cloned individually into the p35S-nYFP vector (Figure 4a). After co-infiltration of individual mutant MP constructs with pCP-cYFP into *N. benthamiana* leaves, YFP fluorescence was examined in the leaves co-expressing one of the five mutant MPs and the CP-cYFP (Figures 4b and S2). The result showed that co-expression of MP^{T107A}-nYFP and CP-cYFP in *N. benthamiana* leaf cells failed to produce YFP fluorescence (Figure 4b). To further confirm the above findings, we constructed plasmid MP^{T107A}-cYFP and co-infiltrated it with pCP-nYFP into *N. benthamiana* leaves. As expected, no YFP fluorescence was observed (Figure S2). We then substituted the other six conserved amino acid residues (E50, S51, Y69, S78, D134, E161) with alanine to produce $pMP^{ES5051AA}$-nYFP,



Coefficient	Values
Pearson's correlation coefficient	0.75259
Manders's overlap coefficient	0.98139
ColocCoeff1:EGFP	0.99893
ColocCoeff2:AF594	1
WColocCoeff1:EGFP	0.99933
WColocCoeff2:AF594	1

FIGURE 2 Colocalization of MP-RFP and CP-YFP in *Nicotiana benthamiana* leaf cells. *N. benthamiana* leaves were agro-infiltrated with *Agrobacterium* cultures carrying plasmid pMP-RFP, pCP-YFP, or both. (a) Confocal images showing colocalization of MP-RFP and CP-YFP in cells. Merged image shows fluorescent punctate signals near the cell walls. Images were taken at 2 days postinfiltration under a LSM710 laser scanning microscope. Bar = 20 μm. (b) Enlarged images from (a) show the colocalization of MP-RFP and CP-YFP. Bar = 5 μm. (c) Colocalization of MP-RFP and CP-YFP was confirmed through coefficient value analysis. (d) Images show CP-YFP localization in cell periphery and nucleus of H2B-RFP transgenic *N. benthamiana* leaf cells. Bar = 10 μm. (e) Images show MP-RFP localization in *N. benthamiana* leaf cells. Bar represents 10 μm

pMP^{Y69A}-nYFP, pMP^{S78A}-nYFP, pMP^{D134A}-nYFP, and pMP^{E161A}-nYFP. These mutant fusions were then individually co-expressed with CP-cYFP in *N. benthamiana* leaf cells. In this experiment, YFP fluorescence was observed in cells of all the assayed leaves (Figures 4c and S2). Furthermore, Co-IP assays indicated that MP interacted with CP in *N. benthamiana* leaves, while a very weak band was observed for the combination of MP^{T107A} and CP. These results indicated that residue 107 in the MP is important for the interaction with CP (Figure 4d).

2.4 | MP and CP interaction affects disease symptom development

To determine the biological function caused by the MP and CP interaction during CGMMV infection, we constructed mutant CGMMV^{MPT107A} and inoculated it to *N. benthamiana* plants through agro-infiltration. The CGMMV-inoculated *N. benthamiana* plants showed mosaic symptoms by 11 days postinoculation (dpi), while the CGMMV^{MPT107A}-inoculated plants did not show virus symptoms by 11 dpi (Figure 5a). Western blot and reverse transcription-quantitative PCR (RT-qPCR) analysis results showed that CGMMV viral RNA and CP accumulations were significantly reduced in the upper leaves of the CGMMV^{MPT107A}-inoculated plants compared to that in CGMMV-inoculated plants, indicating that disruption of MP and CP interaction inhibited the systemic infection of CGMMV (Figure 5b,c). Time-course analysis showed that the disease symptoms and CP systemic accumulation were inhibited in CGMMV^{MPT107A}-inoculated *N. benthamiana* plants compared with CGMMV-inoculated plants (Figure S3). We analysed the CP accumulation on the inoculated leaves. Time-course analysis showed that the accumulation levels of CGMMV CP in the CGMMV^{MPT107A}-inoculated leaves were

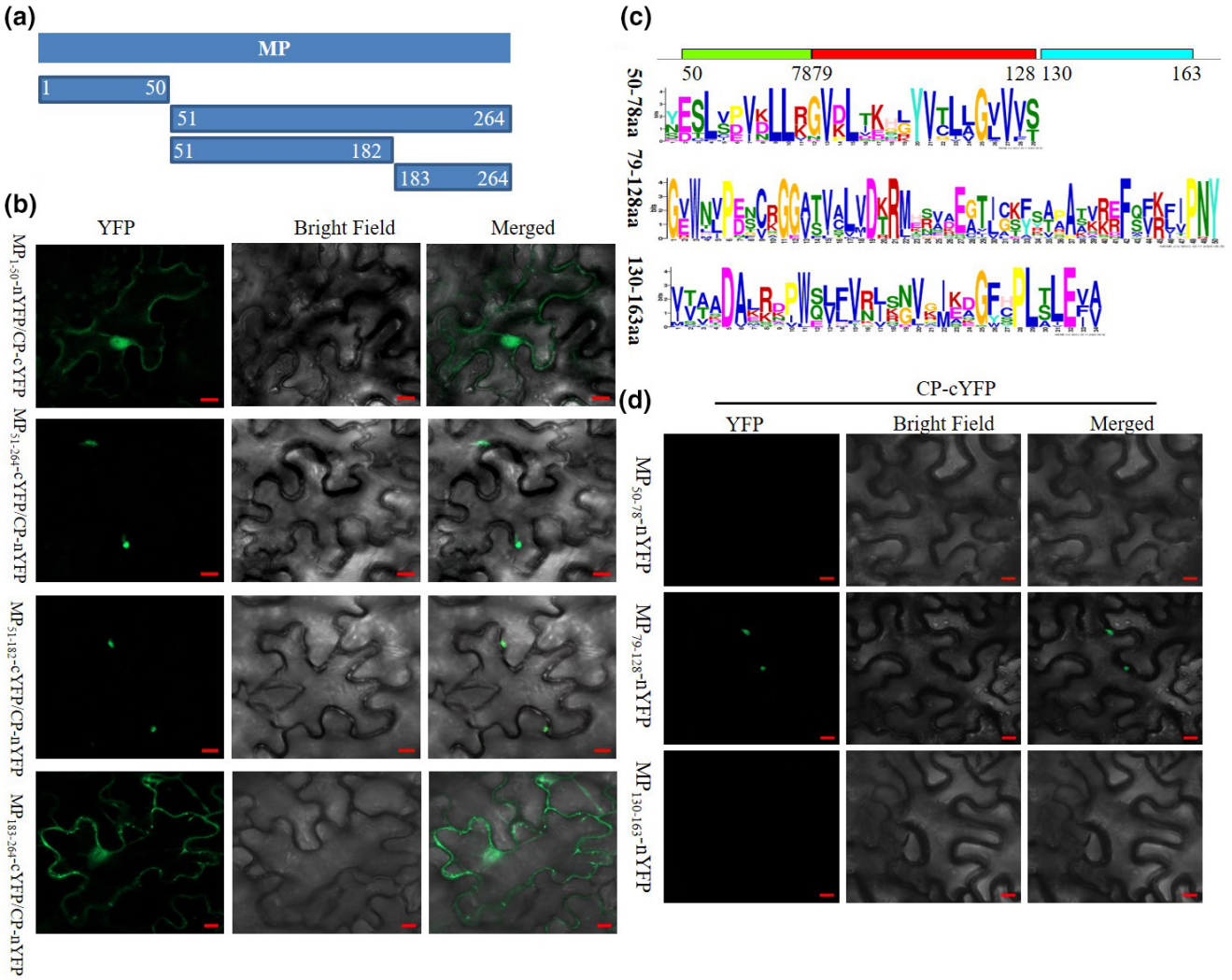


FIGURE 3 Identification of the movement protein–coat protein (MP–CP) interacting domain in cucumber green mottle mosaic virus MP. (a) Schematic representations of the four MP regions. (b) Determination of MP–CP interaction through bimolecular fluorescence complementation (BiFC) using truncated MP mutants. *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium* cultures carrying plasmids pCP-cYFP and $pMP_{1\text{-}50}$-nYFP, pCP-nYFP and $pMP_{51\text{-}264}$-cYFP, pCP-nYFP and $pMP_{51\text{-}182}$-cYFP, pCP-nYFP and $pMP_{183\text{-}264}$-cYFP. The infiltrated leaves were harvested at 2 days post-agro-infiltration (dpai), and examined and imaged under a LSM710 laser scanning microscope. Bar = 10 μm. (c) Schematic representation of the three conserved motifs (positions 50–78, 79–128, and 130–163) was prepared using the MEME online software (https://meme-suite.org/meme/tools/meme). (d) BiFC analysis of the interaction between CP and MP conserved motifs. *N. benthamiana* leaves were infiltrated with *Agrobacterium* cultures carrying plasmids pCP-cYFP and $pMP_{50\text{-}78}$-nYFP, pCP-cYFP and $pMP_{79\text{-}128}$-nYFP, or pCP-cYFP and $pMP_{130\text{-}163}$-nYFP. The infiltrated leaves were harvested at 2 dpai, and examined and imaged under a LSM710 laser scanning microscope. Bar = 10 μm

higher than that in the CGMMMV-inoculated leaves from 4 to 7 dpi (Figure 5d). This finding indicates that the MP–CP interaction promotes CGMMV systemic infection.

2.5 | Conserved amino acids in MP are crucial for viral accumulation

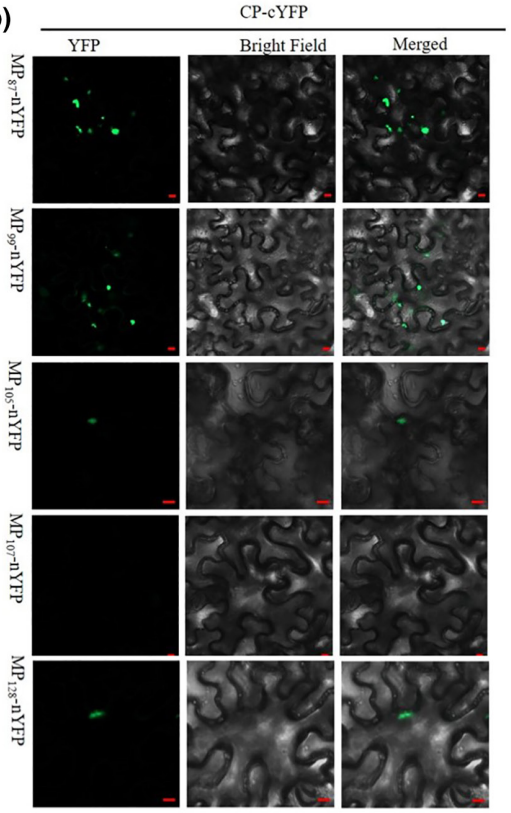
To determine the roles of the other 10 conserved amino acids in MP, which have no impact on MP–CP interaction, on CGMMV accumulation, we constructed nine CGMMV MP mutants. By 14 dpi, $CGMMV^{MPY69A}$, $CGMMV^{MPS78A}$, $CGMMV^{MPC87A}$, and $CGMMV^{MPE161A}$ mutants caused milder mosaic symptoms in *N. benthamiana* plants compared to the wildtype CGMMV. The other five mutant viruses ($CGMMV^{MPES50,51AA}$, $CGMMV^{MPR99A}$, $CGMMV^{MPE105A}$, $CGMMV^{MPY128A}$, and $CGMMV^{MPD134A}$) caused no disease symptoms in the inoculated *N. benthamiana* plants by 14 dpi (Figure 6a). Then we tested CGMMV accumulation in the upper leaves of the plants inoculated with wildtype and CGMMV mutants through western blot and RT-qPCR. Consistent with the disease symptom observation result, mutants $CGMMV^{MPY69A}$, $CGMMV^{MPS78A}$, $CGMMV^{MPC87A}$, and $CGMMV^{MPE161A}$ accumulated to much lower levels than the wildtype CGMMV. CGMMV viral RNA and CP were not detected in the upper leaves of the

(a)

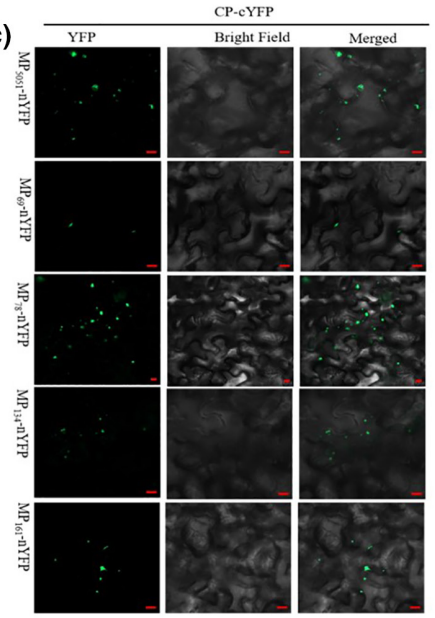
1 MSLSKVSVENSLKPEKFVKISWVDKLLPNYFSILKYLSITDFSVVKAQSYESLVPVKLLR
61 GVDLTKHLYVTLLGVVVSGVWNVPESCRGGATVALVDTRMHSVAEGTICKFSAPATVREF
121 SVRFIPNYSVVAADALRDPWSLFVRLSNVGIKDGFHPLTLEVACLVATTNSIIKKGLRAS
181 VVESVVSSDQSIVLDSLSEKVEPFFDKVPISAAVMARDPSYRSRSQSVGGRGKRHSKPPN
241 RRLDSASEESSSVSFEDGLQSDHT

MP mutant	Susbstitution mutation in CGMMV MP
MP5051	ES5051AA
MP69	Y69A
MP78	S78A
MP87	C87A
MP99	R99A
MP105	E105A
MP107	T107A
MP128	Y128A
MP134	D134A
MP161	E161A

(b)



(c)



(d)

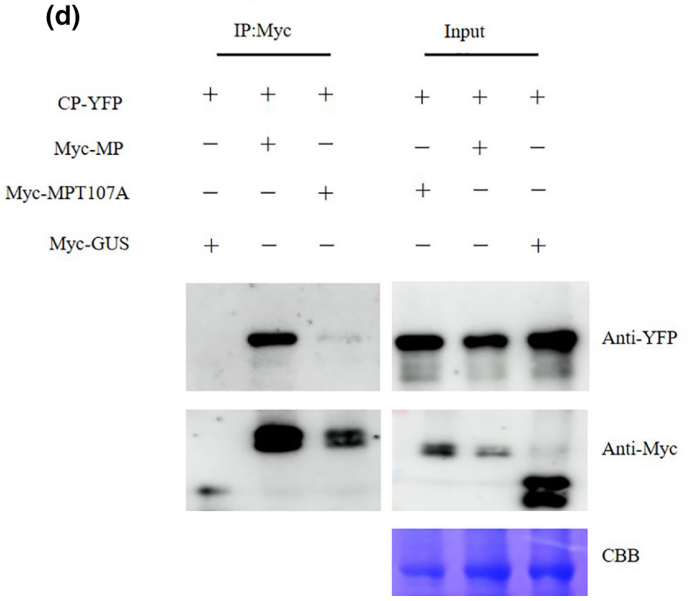


FIGURE 4 The amino acid residue at the position 107 in movement protein (MP) is important for the MP–coat protein (CP) interaction. (a) Amino acid sequence of cucumber green mottle mosaic virus MP (GenBank accession no. KY753929.1). Conserved residues are shown in bold and red letters. Substitutions of the conserved amino acids with alanine in different MP mutants. (b, c) Bimolecular fluorescence complementation analysis of interactions between CP-cYFP and one of the 10 MP-nYFP mutants. *Nicotiana benthamiana* leaves infiltrated with *Agrobacterium* cultures carrying plasmid pCP-cYFP and one of the 10 pMP-nYFP mutants were harvested at 2 days postinfiltration, and examined and imaged under a LSM710 laser scanning microscope. Bar = 10 μm. (d) The interaction analysis of MP–CP and MP^{T107A}–CP in vivo by co-immunoprecipitation. *N. benthamiana* leaves were co-infiltrated with *Agrobacterium tumefaciens* cells harbouring expression vectors to express CP-YFP and Myc-GUS (lane 1 for IP, lane 3 for input), CP-YFP and Myc-MP (lane 2), CP-YFP and Myc-MP^{T107A} (lane 3 for IP, lane 1 for input). Leaf protein extracts were incubated with Myc beads. Samples before (input) and after (IP) immunoprecipitation were analysed by immunoblotting using anti-GFP or anti-Myc antibody

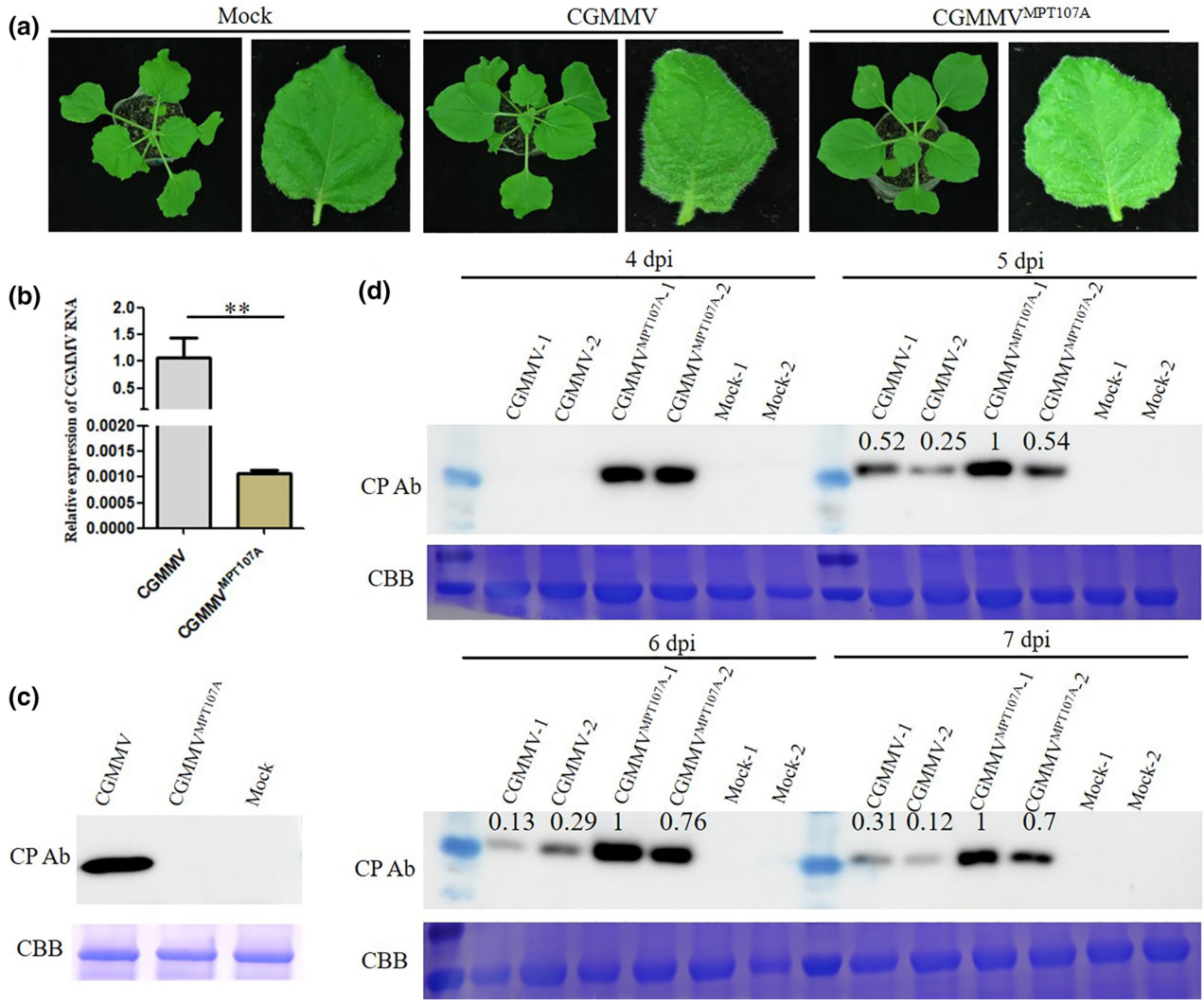


FIGURE 5 Interaction between cucumber green mottle mosaic virus (CGMMV) movement protein (MP) and coat protein (CP) affects viral accumulation and symptom induction. (a) Observations of *Nicotiana benthamiana* plants infected with the wildtype CGMMV and the CGMMV^{MPT107A} mutant at 11 days postinoculation (dpi). (b, c) Detection of CGMMV RNA and CP accumulations in the systemically infected leaves. Upper leaves were harvested from the CGMMV- or CGMMV^{MPT107A}-inoculated plants at 11 dpi. The accumulation of CGMMV RNA was determined through reverse transcription-quantitative PCR (RT-qPCR) using CGMMV-specific primers. The expression level of *NbUBC* was used as an internal control in this experiment. The accumulation of viral CP in these samples was determined through western blot assay using a CP-specific antibody. The Coomassie brilliant blue (CBB)-stained RuBisCO large subunit protein gel was used to show sample loadings. The RT-qPCR result is presented as the means ± *SD* from three biological replicates per treatment. **Significant difference at $p < 0.01$. (d) Time-course analysis of CGMMV CP accumulation in inoculated leaves through western blot assay. The Coomassie brilliant blue-stained RuBisCO large subunit protein gel is used to show sample loadings

plants inoculated with CGMMV^{MPES50,51AA}, CGMMV^{MPR99A}, CGMMV^{MPE105A}, CGMMV^{MPY128A}, and CGMMV^{MPD134A} (Figure 6b,c). By 21 dpi, symptoms on *N. benthamiana* plants inoculated with CGMMV^{MPES50,51AA}, CGMMV^{MPR99A}, CGMMV^{MPE105A}, CGMMV^{MPY128A}, and CGMMV^{MPD134A} were still not obvious (Figure S4). We further analysed the viral RNA accumulation of the five mutants on the inoculated leaves at 6 dpi. The viral accumulation in the leaves inoculated with mutants CGMMV^{MPES50,51AA}, CGMMV^{MPR99A}, CGMMV^{MPE105A}, CGMMV^{MPY128A}, and CGMMV^{MPD134A} was significantly lower than that in the CGMMMV-inoculated leaves (Figure S5), indicating these mutants were possibly impaired in MP movement activity.

2.6 | MP movement activity-associated residue is dispensable for MP and CP interaction

PD localization is a typical MP function. A previous study by Yuan and colleagues indicated that the phenylalanine residue at position 14 in TMV MP is responsible for PD localization (Yuan et al., 2018). In this study, we found the corresponding phenylalanine residue in the CGMMV MP is located at amino acid position 17. We also found the specific motif LXD/X$_{50\text{-}70}$G (Mann et al., 2016) in the CGMMV MP (Figure S6). Inoculation of CGMMV^{MPF17A} and CGMMV^{MPD97A} mutants to leaves of *N. benthamiana* plants did not cause systemic symptoms in the plants by 30 dpi, while the plants inoculated with

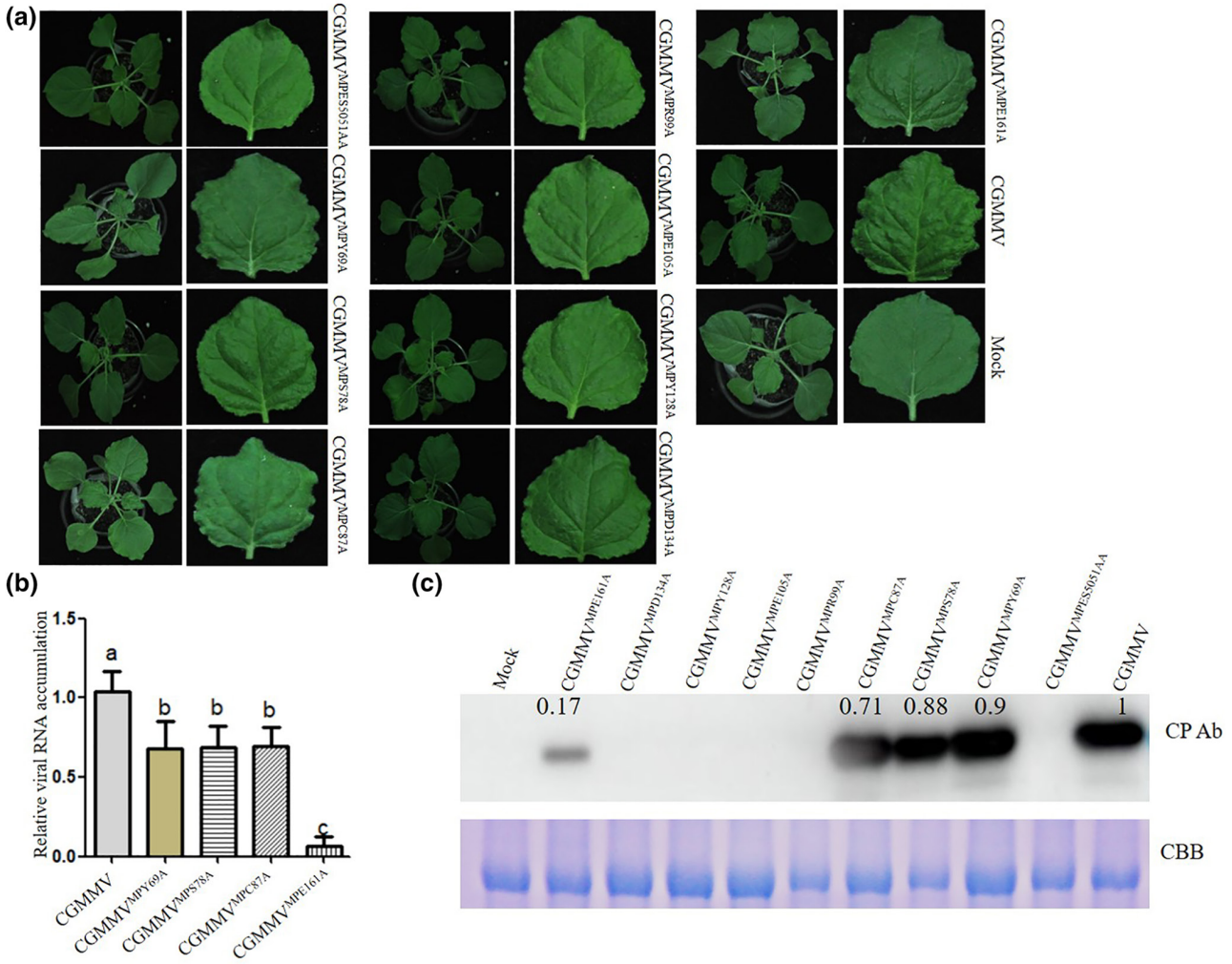


FIGURE 6 Conserved amino acids in cucumber green mottle mosaic virus (CGMMV) movement protein (MP) are crucial for viral accumulation. (a) Observations of *Nicotiana benthamiana* plants inoculated with the wildtype CGMMV and one of the nine CGMMV MP mutants at 14 days postinoculation (dpi). (b, c) Determination of CGMMV RNA and coat protein (CP) accumulation in the upper leaves. Upper leaves of the plants inoculated with the wildtype CGMMV or nine mutants were harvested and analysed at 14 dpi. The accumulation of CGMMV RNA was determined through reverse transcription-quantitative PCR (RT-qPCR) using CGMMV-specific primers. The expression of *NbUBC* was used as an internal control. The accumulation of CGMMV CP in these samples was determined through western blot assay using a CP-specific antibody. The Coomassie brilliant blue (CBB)-stained RuBisCO large subunit protein gel is used to show sample loadings. The RT-qPCR results are presented as the means ± *SD* from three biological replicates per treatment. The statistical significance between the treatments was determined using Student's *t* test ($p < 0.05$)

the wildtype CGMMV showed systemic mosaic symptoms by 10 dpi (Figure 7a). Analyses of systemically infected leaf tissues harvested at 14 dpi from the assayed plants through semiquantitative RT-PCR (sqRT-PCR) and western blot showed that substitution of D97 with alanine significantly reduced the accumulation of CGMMV, and substitution of F17 with alanine abolished the systemic accumulation of CGMMV in upper leaves (Figure 7b,c), indicating the importance of MP movement activity in viral systemic infection. We analysed the viral RNA accumulation on the inoculated leaves at 6 dpi. The viral accumulation levels in leaves inoculated with the CGMMV^{MPF17A} and CGMMV^{MPD97A} mutantswere lower than that in the CGMMMV-inoculated leaves (Figure S7). To investigate the role of MP–CP interaction in MP movement activity, we tested the interaction between one of the two MP mutants and CP through BiFC. The results showed that co-expression of CP-nYFP and MP^{D97A}-cYFP or CP-nYFP and MP^{F17A}-cYFP produced fluorescent punctate signals, similar to that found in the leaves co-expressing CP-nYFP and MP-cYFP, indicating that the residues responsible for MP movement are dispensable for the interaction between CGMMV MP and CP (Figure 7d).

2.7 | Co-expression of MP and CP increases CP accumulation and interaction between MP and CP inhibits SA-related gene expression at an early infection stage

To investigate the role of MP and CP interaction on CGMMV infection, we tested the stabilities of MP and CP after co-expression.

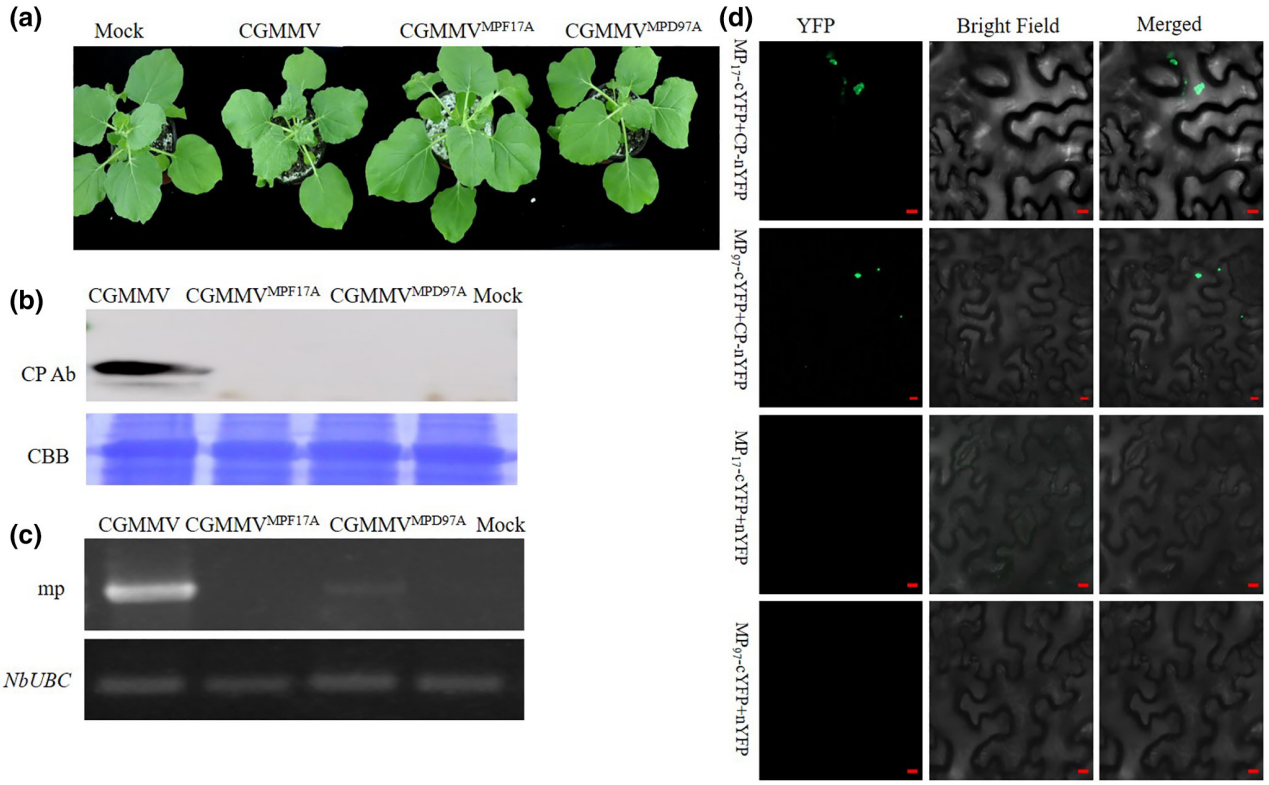


FIGURE 7 Movement protein (MP) movement function was dispensable for the MP–coat protein (CP) interaction. (a) Phenotypes of *Nicotiana benthamiana* plants inoculated with the wildtype cucumber green mottle mosaic virus (CGMMV) and one of the two MP mutants (CGMMV^{MPF17A} and CGMMV^{MPD97A}) at 14 days postinoculation (dpi). (b, c) Detection of CGMMV CP and RNA accumulation in systemically infected leaves. Upper leaves were harvested from the plants inoculated with the wildtype CGMMV and one of the two MP mutants at 14 dpi. The accumulation of CGMMV CP was detected through western blot assay using a CGMMV CP-specific antibody. The Coomassie brilliant blue (CBB)-stained RuBisCO large subunit protein gel was used to show sample loadings. The expression of *NbUBC* in these samples was used as an internal control. The accumulation of CGMMV RNA was detected through semiquantitative RT-PCR using CGMMV-specific primers. (d) Bimolecular fluorescence complementation analysis of the interaction between CP-nYFP and one of the two MP mutants. *N. benthamiana* leaves were co-infiltrated with *Agrobacterium* cultures carrying plasmid pMP^{17}-cYFP and pCP-nYFP, pMP^{97}-cYFP and pCP-nYFP, pMP^{17}-cYFP and pnYFP, or pMP^{97}-cYFP and pnYFP. The infiltrated leaves were harvested and imaged at 2 days post-agro-infiltration under a LSM710 laser scanning microscope. Bar = 10 μm

FLAG-MP and CP-YFP, FLAG-MP and YFP or Myc-GUS and CP-YFP, Myc-MP and CP-YFP were transiently co-expressed in *N. benthamiana* leaves, and the infiltrated leaves were harvested and analysed for protein expression through western blot at 36 h post-agro-infiltration (hpai). The results showed that the expression of MP significantly increased the accumulation of CP (Figure 8a), while the expression of CP slightly suppressed the expression of MP (Figure 8b), suggesting that MP and CP co-expression could affect the accumulation of MP and CP. We further tested the effect of co-expression of MP^{T107A} and CP on the accumulation of MP and CP (Figure 8a,c). The results indicated that MP^{T107A} mutant expression showed a similar effect on CP accumulation as MP, indicating that the regulation of CP accumulation by MP was possibly not directly correlated with the interaction. A previous study using TMV showed that the CP of TMV can regulate the expression of SA-responsive genes, which are required for TMV long-distance movement (Conti et al., 2012; Venturuzzi et al., 2021). To evaluate the interaction on the expression of SA-related genes in the *N. benthamiana* leaves, we analysed the expression levels of *NPR1*and *PR1a*. The results showed that the expression levels of *NPR1* and *PR1a* were significantly up-regulated in the CGMMV^{MPT107A}-inoculated systemic leaves compared with CGMMV and healthy control at 4 dpi. At 5 dpi, the expression levels of *PR1a* in both CGMMV-inoculated and systemic leaves were significantly lower than in CGMMV^{MPT107A} and control. This finding indicates that CGMMV MP–CP interaction could inhibit the SA-associated defence response at an early infection stage (Figure S8).

3 | DISCUSSION

Plant viruses have small genomes, and therefore encode only a few proteins with multiple functions, important for different steps of viral infections. Here we present evidence to show that CGMMV MP can interact with CP, and this interaction is possibly not linked to the movement ability of MP. The interaction is, however, involved in systemic infection, possibly through suppression of host antiviral defence.

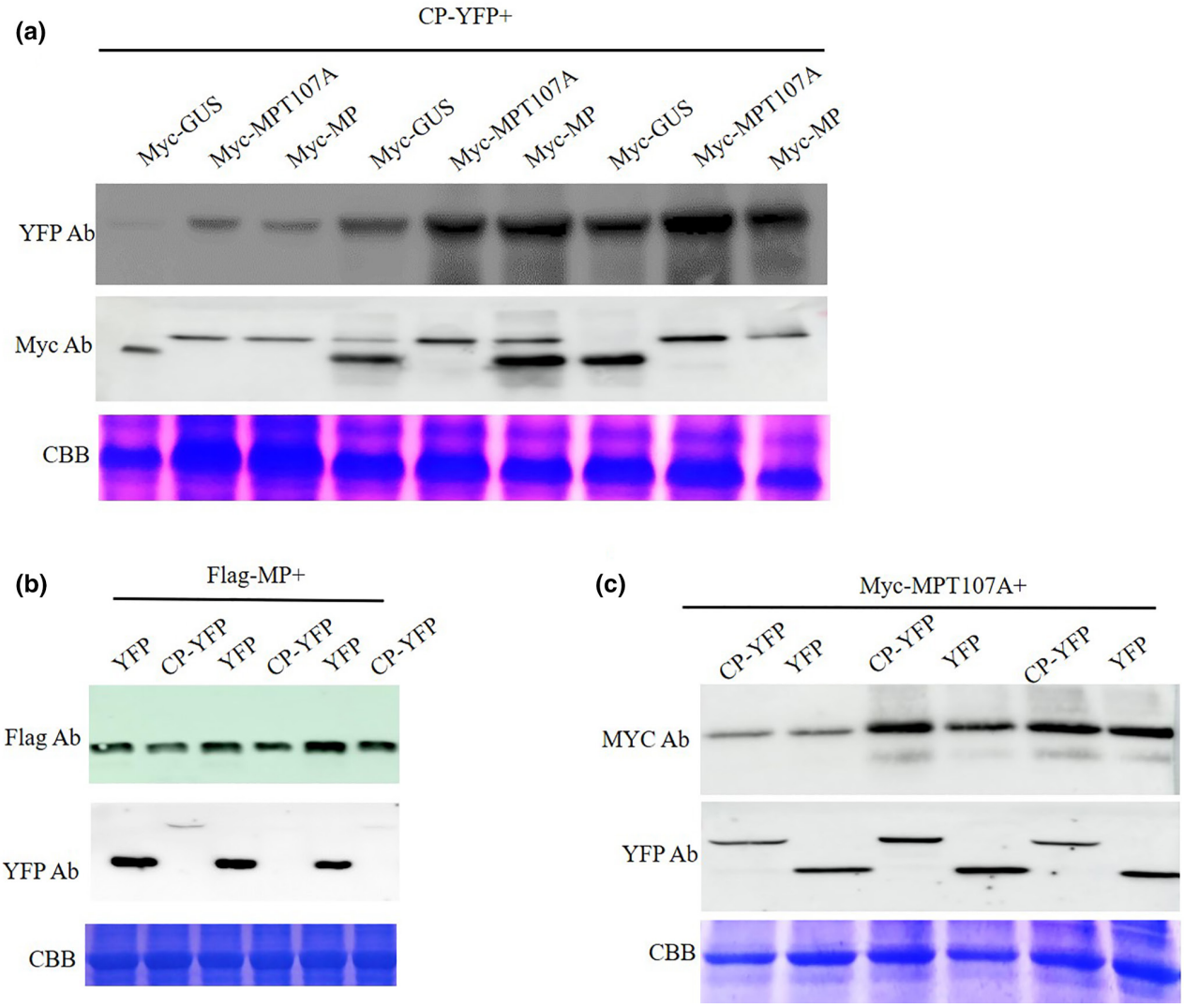


FIGURE 8 Effects of co-expression of movement protein (MP) and coat protein (CP), MP^{T107A} and CP on the accumulation of CP or MP. *Agrobacterium* cultures harbouring Myc-GUS and CP-YFP, Myc-MP and CP-YFP, Myc-MP^{T107A} and CP-YFP, FLAG-MP and CP-YFP, FLAG-MP and YFP were infiltrated individually into *Nicotiana benthamiana* leaves. The accumulation of Myc-GUS, Myc-MP, Myc-MP^{T107A}, FLAG-MP, CP-YFP, and YFP in these infiltrated leaf tissues was determined through western blot assay using a GFP-, Myc-, or a FLAG-specific antibody at 36 h postinfiltration. The Coomassie brilliant blue (CBB)-stained RuBisCO large subunit protein gel was used to show sample loadings. (a) The effect of MP and MP^{T107A} expression on the CP accumulation. The accumulation of CP-YFP in these samples was detected through western blot assay using a GFP-specific antibody. The accumulation of Myc-MP, Myc-MP^{T107A} and Myc-GUS in these samples were detected through western blot assay using an anti-Myc antibody. (b) The effect of CP expression on the MP accumulation. The accumulation of CP-YFP and YFP in these samples was detected through western blot assay using a GFP-specific antibody. The accumulation of FLAG-MP in these samples was detected through western blot assay using a FLAG-specific antibody. (c) The effect of CP expression on the MP^{T107A} accumulation. The accumulation of CP-YFP and YFP in these samples was detected through western blot assay using a GFP-specific antibody. The accumulation of Myc-MP^{T107A} in these samples was detected through western blot assay using a Myc-specific antibody

The TMV CP^{T42W} mutant virus is known to inhibit MP production or accumulation, while the wildtype CP shows the opposite effect (Bendahmane et al., 2002). TMV CP and MP interaction was observed in cells in the presence of CP-nYFP and MP-cYFP, but not in the presence of CP-cYFP and MP-nYFP (Bazzini et al., 2007). In our study, both combinations (i.e., CP-cYFP and MP-nYFP, and CP-nYFP and MP-cYFP) produced strong YFP fluorescence. In addition, we have found that the CGMMV MP and CP interaction site appeared as punctate signals in the cells, which is different from the interaction of TMV MP and CP.

According to previous reports on the MP of TMV, we have found two corresponding amino acid residues in the CGMMV MP, and these two conserved amino acid residues are phenylalanine (F) and aspartic acid (D) at positions 17 and 97, respectively (Yuan et al., 2018). As expected, substitution of F17 or D97 with alanine inhibited CGMMV systemic infection, but not the interaction between the mutant MPs and the CP. This finding indicates that the MP–CP interaction probably exerts other functions without impact on the movement activity of MP. Infection assays using non-CP-interacting MP mutant virus

showed that the MP–CP interaction is possibly independent of MP movement activity. It is noteworthy that all the conserved amino acids of MP selected for mutational analyses without impact on the MP–CP interaction are crucial for CGMMV infection.

As reported previously, TMV MP is the second protein to accumulate during virus infection and is required for cell-to-cell movement through interaction with PD (Heinlein, 2015). After cell-to-cell movement to reach vascular tissues, TMV systemic spread is initiated with the participation of viral CP (Conti et al., 2017). How MP–CP interaction coordinates the switch from cell-to-cell movement to systemic infection is unknown. In our study, we further confirmed that the movement function of MP is indispensable for viral infection via two CGMMV MP-specific mutants. We also found that the movement function of MP was not linked to its interaction with CP. Analysis of this interaction using MP deletion mutants further confirmed the above finding. The N-terminal 50 amino acids in TMV MP have been reported as the TMV MP PD localization signal (Yuan et al., 2016) but are not essential for the MP and CP interaction.

In this study, we analysed the effect of CGMMV MP and CP interaction on their accumulation and CGMMV infection. Our results showed that the expression of both MP and MP^{T107A} can positively regulate the expression of CP, indicating that the regulation of CP accumulation by MP was possibly not directly correlated with the interaction. Previous studies had shown that TMV CP can repress the host defence response (Callaway et al., 2001; Conti et al., 2017; Venturuzzi et al., 2021). Negative regulation of the SA signalling pathway by the CP of TMV is required for viral systemic movement (Venturuzzi et al., 2021). Based on the inhibition of the SA-associated defence response at an early infection stage by MP–CP interaction, we speculate that the suppression of host antiviral defence through the MP–CP interaction possibly facilitates viral systemic movement.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant growth and virus inoculation

Wildtype and H2B-transgenic *N. benthamiana* plants were grown in pots inside a growth room maintained at 25°C, 16 h light/8 h dark photoperiod, and 60% humidity. CGMMV was from a previously reported source (Liu et al., 2017). For agro-infiltration, *Agrobacterium tumefaciens* GV3101 carrying individual infectious viral clones was suspended in an infiltration buffer (10 mM $MgCl_2$, 10 mM MES, and 200 μM acetosyringone, pH 5.6) to OD_{600} = 1, incubated at room temperature for 2–4 h, and then infiltrated into leaves of *N. benthamiana* using 1-ml needleless syringes.

4.2 | Amino acid sequence alignment

Sequences of 20 tobamovirus MPs were from the database at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) (O'Leary et al., 2016). Multiple sequence alignment was performed using DNAMAN software. Motifs in MP partial sequences were predicted using MEME software (https://meme-suite.org/meme/tools/meme).

4.3 | Plasmid construction

Detailed information about the constructions of plasmids is listed in the Table S1. All the resulting constructs were sequenced before use.

For BiFC analysis, coding sequences of CGMMV *MP*, *CP*, and *P2* genes were individually amplified and cloned into the entry vector pGWC via the in-fusion method (Zhou et al., 2018). The sequence representing the MP amino acids 1–50 (referred to as MP1–50), 51–182 (MP51–182), 50–78 (MP50–78), 51–264 (MP51–264), 79–128 (MP79–128), 130–163 (MP130–163), 183–264 (MP183–264), MP^{F17A}, $MP^{ES50,51AA}$, MP^{Y69A}, MP^{S78A}, MP^{C87A}, MPD97A, MP^{R99A}, MP^{E105A}, MP^{T107A}, MP^{Y128A}, MP^{D134}A, and MP^{E161A} were amplified individually and cloned into the Gateway Entry vector pGWC using ClonExpress II One Step Cloning Kit as instructed (Vazyme). The resulting clones were then used to clone into the Gateway vector p35S-nYFP and p35S-cYFP vectors through the LR reaction.

For subcellular localization and transient expression assays, MP and CP were cloned into the Gateway vector RFP and pEarleyGate101 to produce plasmid pMP-RFP and pCP-YFP, respectively. The MP coding region was also PCR amplified and cloned into the expression vector pGDFlag to produce pGDFlagMP.

To generate CGMMV MP mutants, the codons of E50, S51, Y69, S78, C87, R99, E105, T107, Y128, D134, and E161 were substituted with alanine (A) through overlapping PCR to produce plasmids $pCGMMV^{MPES50,51AA}$, $pCGMMV^{MPY69A}$, $pCGMMV^{MPS78A}$, $pCGMMV^{MPC87A}$, $pCGMMV^{MPR99A}$, $pCGMMV^{MPE105A}$, $pCGMMV^{MPT107A}$, $pCGMMV^{MPY128A}$, $pCGMMV^{MPD134A}$, and $pCGMMV^{MPE161A}$.

4.4 | Confocal laser scanning microscopy

For BiFC and colocalization assays, *Agrobacterium* cultures carrying the corresponding plasmids were individually infiltrated into the leaves of *N. benthamiana* plants as described in the figure legends. The infiltrated leaves were detached at 48 hpai. Fluorescence signals were examined under an inverted confocal laser scanning microscope (LSM 710; Zeiss). The YFP and RFP were excited at 514 and 561 nm wavelengths, respectively.

4.5 | Western blot analysis

The agro-infiltrated leaves were harvested, and total protein was extracted from these leaves (0.2 g per sample) using an extraction buffer (20% glycerol, 20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM PMSF, and 1× protease inhibitor cocktail; Sigma-Aldrich). The extracted protein samples were separated in SDS-polyacrylamide

gels through electrophoresis, followed by transferring them to nitrocellulose membranes. The membranes were then probed with a polyclonal anti-CP antibody, an anti-GFP antibody (TransGen), an anti-Myc antibody (Sigma-Aldrich) or an anti-FLAG antibody (Sigma-Aldrich), followed by a horseradish peroxidase-conjugated secondary antibody. The detection signals were developed by addition of an ECL reagent (Vazyme) as instructed.

4.6 | Quantitative and semiquantitative RT-PCR

Total RNA was extracted from the harvested *N. benthamiana* leaf samples using TRIzol reagent (Invitrogen) and treated with RNase-free DNase I. First-strand cDNA was synthesized using 1 μg of total RNA (per 20 μl reaction), random primers, and M-MLV reverse transcriptase. Ten-fold diluted cDNA products and the SYBR Green master mix (Takara) were used for qPCR on an Eppendorf Real-Time PCR system. The expression of *NbUBC* was used as an internal control. All the RT-qPCR primers are listed in Table S1. The relative RNA level in each sample was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

For sqRT-PCR, a 307 bp fragment representing a partial sequence *NbUBC* and a 795 bp fragment representing the CGMMV *MP* gene was amplified. The expression level of *NbUBC* was used as an internal control. The sqRT-PCR was performed as follows: predenaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s; followed by the final extension at 72°C for 10 min. The resulting products were analysed in 1% agarose gels through electrophoresis.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the finding of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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