The conserved aromatic residue W\textsuperscript{122} is a determinant of potyviral coat protein stability, replication, and cell-to-cell movement in plants

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Abstract

Coat proteins (CPs) play critical roles in potyvirus cell-to-cell movement. However, the underlying mechanism controlling them remains unclear. Here, we show that substitutions of alanine, glutamic acid, or lysine for the conserved residue tryptophan at position 122 (W\textsuperscript{122}) in tobacco vein banding mosaic virus (TVBMV) CP abolished virus cell-to-cell movement in \textit{Nicotiana benthamiana} plants. In agroinfiltrated \textit{N. benthamiana} leaf patches, both the CP and RNA accumulation levels of three W\textsuperscript{122} mutant viruses were significantly reduced compared with those of wild-type TVBMV, and CP accumulated to a low level similar to that of a replication-deficient mutant. The results of polyprotein transient expression experiments indicated that CP instability was responsible for the significantly low CP accumulation levels of the three W\textsuperscript{122} mutant viruses. The substitution of W\textsuperscript{122} did not affect CP plasmodesmata localization or virus particle formation; however, the substitution significantly reduced the number of virus particles. The wild-type TVBMV CP could complement the reduced replication and abolished cell-to-cell movement of the mutant viruses. When the codon for W\textsuperscript{122} was mutated to that for a different aromatic residue, phenylalanine or tyrosine, the resultant mutant viruses moved systemically and accumulated up to 80% of the wild-type TVBMV level. Similar results were obtained for the corresponding amino acids of W\textsuperscript{122} in the watermelon mosaic virus and potato virus Y CPs. Therefore, we conclude that the aromatic ring in W\textsuperscript{122} in the core domain of the potyviral CP is critical for cell-to-cell movement through the effects on CP stability and viral replication.

KEYWORDS
cell-to-cell movement, coat protein, Potyvirus, replication, stability, Tobacco vein banding mosaic virus

1 | INTRODUCTION

The genus Potyvirus, belonging to the family Potyviridae, is the largest genus of plant RNA viruses, consisting of more than 160 species (Wylie et al., 2018). Many potyviruses cause severe economic losses in crop production (García et al., 2014; Scholthof et al., 2011; Valkonen, 2007). The potyvirus genome is a positive-sense single-stranded RNA molecule with 9,400 to 11,000 nucleotides.

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The potyviral genomic RNA has a polyadenylated tail at its 3’ end and covalently links with a genome-linked protein (VPg) at its 5’ end (Tavert-Roudet et al., 2017; Wylie et al., 2018). The potyvirus genome encodes two polyproteins that are processed into 11 mature proteins by three self-encoded proteinases (Chung et al., 2008; Olspert et al., 2015; Revers & García, 2015; Rodamilans et al., 2015).

Cell-to-cell movement is a critical step for plant viruses to establish systemic infection (Ritzenzhaler, 2011; Schoelz et al., 2011). To move between cells, plant viruses take advantage of plasmodesmata (PD), which are microscopic channels for plant communication between adjacent cells. Potyviruses do not encode a specific movement protein. However, the second 6 kDa protein (6K2) (Grangeon et al., 2013; Jiang et al., 2015), the third protein (P3) (Chai et al., 2020), the frame-shift protein P3N–PIPO produced by transcriptional slippage (Cheng et al., 2017; Geng et al., 2015; Olspert et al., 2015; Rodamilans et al., 2015; Wei et al., 2010), the cylindrical inclusion protein (CI) (Carrington et al., 1998; Deng et al., 2015; Movahed et al., 2017; Roberts et al., 1998; Rodríguez-Cerezo et al., 1997), and the coat protein (CP) (Dolja et al., 1994, 1995) are known to be involved in potyviral cell-to-cell movement.

Potyviral CPs contain three domains: the N-terminal domain with 17–78 residues (the numbers vary among viruses), the core domain with 213–218 residues (starting with the KDK/D residues at the N-terminus and ending with the TER/H residues at the C-terminus) (Shukla et al., 1997), and the C-terminal domain with 17–21 residues. Deletion of the N- or C- terminal domains of the tobacco etch virus (TEV) CP reduces its cell-to-cell movement in plants (Dolja et al., 1994, 1995). Alteration in the net charge of the N-terminal domain of the zucchini yellow mosaic virus CP, or mutation of the charged arginine residue at position 245 (R245), histidine at position 246 (H246), or aspartic acid at position 250 (D250), which are at the border between the core and the C-terminal domains of the soybean mosaic virus CP, nearly abolishes viral cell-to-cell movement in plants (Kimalov, 2004; Seo et al., 2013). The phosphorylation sites in the T242–TSEED247 motif in CP are critical for the replication of potato virus A (Ivanov et al., 2003; Löhmus et al., 2017). Mutation of the residues in serine (S122), R154, or D198 in the TEV CP completely abolishes TEV particle formation and cell-to-cell movement, suggesting that TEV moves between cells in the form of viral particles (Dolja et al., 1994, 1995). Although the core domain of the potyvirus CP is known to be involved in the viral cell-to-cell movement, the roles of the conserved amino acid residues in this domain in viral cell-to-cell movement remain largely unknown.

In this study, we determined that the aromatic ring of the conserved tryptophan residue at position 122 (W122) in the potyvirus CP core domain plays an essential role in viral cell-to-cell movement through maintaining viral replication and CP stability.

## RESULTS

### 2.1 Mutations of W122 abolish TVBMV cell-to-cell and systemic movement in Nicotiana benthamiana plants

To investigate the effects of conserved amino acid residues in the TVBMV CP on virus infection, we first aligned the CP sequences of 139 potyviruses and identified the completely conserved aromatic residue tryptophan (W) at position 122 (W122), numbered according to the TVBMV CP sequence (Figure S1). We then mutated the codon for W122 in the pCamTVBMV-GFP, an TVBMV infectious clone carrying the green fluorescent protein (GFP) gene, to the codons for residue alanine (A), glutamic acid (E), or lysine (K) to produce the plasmids pCamTVBMV-W122A-GFP, pCamTVBMV-W122E-GFP, and pCamTVBMV-W122K-GFP, respectively (Figure 1a). Agrobacterium cultures carrying pCamTVBMV-GFP or one of the three mutant plasmids were individually infiltrated into two expanded leaves of each assayed N. benthamiana plant to produce the viruses TVBMV-GFP, TVBMV-W122A-GFP, TVBMV-W122E-GFP, and TVBMV-W122K-GFP, respectively. At 7 days postagroinfiltration (dpai), the TVBMV-GFP-infected plants showed mosaic and epinasty symptoms in their systemically infected leaves, but the three mutant viruses did not (Figure 1b). When the infiltrated plants were examined under a UV light, the TVBMV-GFP-infected plants emitted a strong green fluorescence in their infiltrated and systemically infected leaves. This green fluorescence was only observed in the leaves infiltrated with pCamTVBMV-W122A-GFP, pCamTVBMV-W122E-GFP, and pCamTVBMV-W122K-GFP, respectively (Figure 1b). The results of the reverse transcription (RT)-PCR and western blot assays showed that TVBMV RNA and CP had accumulated in the systemically infected leaves of the TVBMV-GFP-inoculated plants, but not in the systemically infected leaves of the plants inoculated with one of the three mutant viruses (Figure 1c,d). To validate the importance of W122 in TVBMV systemic movement, we changed the codon of nonconserved residues arginine at position 62 (R62) to that for A, cysteine (C), or E; serine at position 92 (S92) to that for A, aspartic acid (D), or histidine (H); and K at position 246 (K246) to that for A, C, or E in pCamTVBMV-GFP. These mutant plasmids were individually infiltrated into N. benthamiana leaves and these mutations did not inhibit viral systemic infection (Figure S2), indicating that W122 in the CP core domain was crucial for TVBMV systemic infection in N. benthamiana plants.

Next, we investigated the role of W122 in TVBMV cell-to-cell movement. Agrobacterium cultures carrying pCamTVBMV-GFP or one of its three W122 mutant plasmids were diluted to OD600 = 0.0001 and individually infiltrated into N. benthamiana leaves. GFP fluorescence from TVBMV-GFP was observed in clusters of multiple cells at 132 hr postagroinfiltration (hpai). However, the GFP fluorescence from TVBMV-W122A-GFP, TVBMV-W122E-GFP, or TVBMV-W122K-GFP was confined to single leaf cells (Figure 1e,f), indicating that W122 was also crucial for TVBMV cell-to-cell movement.

### 2.2 Mutations of W122 inhibit TVBMV replication

Cell-to-cell movement and replication are two important and interconnected steps during potyvirus infection (Chai et al., 2020; Cui & Wang, 2016). To determine whether W122 has a role in TVBMV replication, we infiltrated N. benthamiana leaves with pCamTVBMV-GFP or one of the three W122 mutant plasmids. Plants infiltrated
FIGURE 1 Conserved residue W$^{122}$ in tobacco vein banding mosaic virus coat protein (TVBMV CP) is crucial for viral cell-to-cell and systemic movement. (a) A schematic diagram showing the genome organization of TVBMV in pCamTVBMV-GFP. The N- and C-terminal domains of TVBMV CP are in grey, and the core domain is in black. The relative position of W$^{122}$ is indicated with a red arrowhead. The site-directed mutagenized plasmids (left column) and viruses (right column) are shown in the red-lined box. pCamTVBMV-GFP, pCamTVBMW$^{122A}$-GFP, pCamTVBMW$^{122E}$-GFP, and pCamTVBMW$^{122K}$-GFP plasmids were individually agroinfiltrated into Nicotiana benthamiana plants. (b) Symptoms (upper panel) and green fluorescent protein (GFP) green fluorescence (lower panel) in various assayed N. benthamiana plants at 7 days postagroinfiltration (dpai) are shown. (c) and (d) Accumulation levels of TVBMV RNA and CP in the systemic leaves of the assayed plants were determined at 7 dpai through reverse transcription-PCR and western blot assay, respectively. The expression of the Nbef1a gene was used as an internal control during RT-PCR. Ab: antibody. The Coomassie brilliant blue-stained RuBisCO large subunit protein (Rubi) was used to show sample loadings. (e) and (f) Cell-to-cell movement of TVBMV-GFP and its three W$^{122}$ mutants in N. benthamiana leaves at 132 hr postagroinfiltration (hpa). The pictures were taken under a confocal microscope at 132 hpa. Because each TVBMV-GFP infection focus contained too many cells with GFP fluorescence, its number is not shown in (f). The values are presented as means ± SD from 30 infection foci per treatment. Statistical significance between treatments was determined using Duncan’s multiple range test ($p$ < .05)
with pCamTVBMV\textsuperscript{WT}\textsubscript{GDD}-GFP (produce replication-deficient mutant TVBMV\textsuperscript{WT}\textsubscript{GDD}-GFP; Geng et al., 2017) or pCamTVBMV\textsuperscript{CPSTOP}-GFP (producing TVBMV\textsuperscript{CPSTOP}-GFP that did not produce CP) were used as controls. The infiltrated leaves were harvested at 60 hpi and analysed for GFP or CP accumulation using enzyme-linked immunosorbent assay (ELISA). The results showed that the accumulation levels of CP in the TVBMV\textsuperscript{W122A}\textsubscript{GFP}, TVBMV\textsuperscript{W122E}\textsubscript{GFP}, or TVBMV\textsuperscript{W122K}\textsubscript{GFP}-inoculated leaves were significantly reduced compared with that of the TVBMV-GFP-inoculated leaves. The accumulation levels of GFP in the TVBMV\textsuperscript{W122A}\textsubscript{GFP} or TVBMV\textsuperscript{CPSTOP}-GFP-inoculated leaves were the lowest (Figure 2a). The accumulation levels of CP in the TVBMV\textsuperscript{W122A}\textsubscript{GFP}, TVBMV\textsuperscript{W122E}\textsubscript{GFP}, and TVBMV\textsuperscript{W122K}\textsubscript{GFP}-inoculated leaf tissues were also significantly reduced compared with that of the TVBMV-GFP-inoculated leaf tissues and reached a similar level to that of mutant TVBMV\textsuperscript{NIb}−GFP, GFP-inoculated leaf tissues (Figure 2b). Similar results were obtained from three biological replicates per treatment. Statistical significance between treatments was determined using Duncan’s multiple range test (p < 0.05).

2.3 | W\textsuperscript{122} is crucial for TVBMV CP stability

To investigate why the three W\textsuperscript{122} mutants accumulated significantly less CP than TVBMV-GFP, we constructed two vectors to transiently express polyprotein, NlaPro:HA-Nlb:GFP:CP and NlaPro:HA-Nlb:GFP:CP\textsuperscript{P192A}, in N. benthamiana leaves through agroinfiltration (Figure 3a). Each of the polyproteins could be self-cleaved by NlaPro into four mature proteins: NlaPro, HA-Nlb, GFP, and CP (or the mutant CP\textsuperscript{P192A}). N. benthamiana leaves were infiltrated with mixed Agrobacterium cultures harbouring pCamNlaPro:HA-Nlb:GFP:CP and pBinP19, or pCamNlaPro:HA-Nlb:GFP:CP\textsuperscript{P192A} and pBinP19. By 4 dpi, and the intensity of GFP fluorescence in the pCamNlaPro:HA-Nlb:GFP:CP and pBinP19 coinfiltrated leaf patches was similar to that in the pCamNlaPro:HA-Nlb:GFP:CP and pBinP19 coinfiltrated leaf patches (Figure 3b). Western blot results showed that the accumulation levels of HA-Nlb and GFP in the pCamNlaPro:HA-Nlb:GFP:CP\textsuperscript{P192A} and pBinP19 coinfiltrated leaf patches were similar to those in the pCamNlaPro:HA-Nlb:GFP:CP and pBinP19 coinfiltrated leaf patches (Figure 3c). However, the accumulation level of CP in the pCamNlaPro:HA-Nlb:GFP:CP\textsuperscript{P192A} and pBinP19 coinfiltrated leaf patches was only one-ninth of that in the pCamNlaPro:HA-Nlb:GFP:CP and pBinP19 coinfiltrated leaf patches (Figure 3c). These results suggest that mutation of W\textsuperscript{122} affects CP stability.

To further investigate the stability of the W\textsuperscript{122} mutant CP, we constructed pCamGFP-TVBMVCP and pCamGFP-TVBMVCP\textsuperscript{P192A} to express a GFP-TVBMVCP fusion and a GFP-TVBMVCP\textsuperscript{P192A} fusion, respectively (Figure 3d). N. benthamiana leaves were infiltrated with mixed Agrobacterium cultures harbouring pCamGFP-TVBMVCP and pBinP19, or pCamGFP-TVBMVCP\textsuperscript{P192A} and pBinP19. The infiltrated leaves were photographed and analysed at different times. The results showed that the intensity of GFP fluorescence in the leaf patches coexpressing GFP-TVBMVCP and P19 continued to increase from 30 to 120 hpi, whereas the intensity of GFP fluorescence in the leaf patches coexpressing GFP-TVBMVCP\textsuperscript{P192A} and P19 remained low (Figure 3e,f). We also constructed plasmids pCamTVBMVCP\textsuperscript{WT}}
and pCamTVBMVCP<sub>W122A</sub> to transiently express TVBMVCP<sup>WT</sup> and TVBMVCP<sub>W122A</sub> in <i>N. benthamiana</i> leaves. Western blot results showed that, at 4 dpai, the accumulation levels of TVBMVCP<sup>WT</sup> and TVBMVCP<sub>W122A</sub> in the assayed leaf patches agreed with the results for GFP shown in Figure 3e,f (Figure S4). The expression of His-TVBMVCP and His-TVBMVCP<sub>W122A</sub> in <i>Escherichia coli</i> cells also showed that, at 4 dpai, the accumulation levels of TVBMVCP<sup>WT</sup> and TVBMVCP<sub>W122A</sub> in the assayed leaf patches agreed with the results for GFP shown in Figure 3e,f (Figure S4).
indicated that the mutation of $W^{122}$ to A affected the stability of TVBMV CP (Figure S5). All these results indicate that mutation of $W^{122}$ affects CP stability.

2.4 The mutation of $W^{122}$ does not affect TVBMV particle formation

Previous studies have shown that virus particle formation is vital during potyviral cell-to-cell movement (Dolja et al., 1994). To determine whether the mutation of $W^{122}$ could affect virus particle formation, we expressed TVBMV-GFP, TVBMV$^{W122A}$-GFP, TVBMV$^{W122E}$-GFP, and TVBMV$^{W122K}$-GFP in N. benthamiana leaves. At 5 dpi, the infiltrated leaves were collected and used for virus particle purification. When the purified samples were negatively stained with 2% uranyl acetate and examined under a transmission electron microscope, virus particles similar to that of TVBMV-GFP were observed in the TVBMV$^{W122A}$, GFP, TVBMV$^{W122E}$-GFP, and TVBMV$^{W122K}$-GFP purified samples, indicating that the mutation of $W^{122}$ did not change the ability of CP to encapsidate viral RNA (Figure 4a). However, the numbers of virus particles in the TVBMV$^{W122A}$-GFP, TVBMV$^{W122E}$-GFP, and TVBMV$^{W122K}$-GFP purification samples were significantly reduced compared with that in the TVBMV-GFP purification sample (Figure 4b).

2.5 The mutation of $W^{122}$ does not affect the ability of CP to target PD

Potyviral CP, P3N-PIPO, and CI are known to target PD in cell walls and are necessary for the potyviral cell-to-cell movement (Wei et al., 2010). To investigate the effects of $W^{122}$ on TVBMV CP subcellular localization, we coinfiltrated N. benthamiana leaves with mixed Agrobacterium cultures harbouring combinations of four constructs, including pCamTVBMV, pCamP3N-PIPO, pCamCI-DsRed, and pCamGFP-TVBMVCP or pCamGFP-TVBMVCPO$^{W122A}$. At 48 hpi, analyses of infiltrated leaf patches under a confocal microscope showed that GFP-TVBMVCP and GFP-TVBMVCPO$^{W122A}$ could colocalize with CI-DsRed in the cell periphery targeted to PD (Figure 5). Our previous results showed that TVBMV CI can localize at PD in the presence of TVBMV P3N-PIPO (Geng et al., 2015). These results suggest that GFP-TVBMVCP and GFP-TVBMVCPO$^{W122A}$ could target PD with CI-DsRed and P3N-PIPO during TVBMV infection.

2.6 Wild-type CP, but not the W122A mutant CP, rescue TVBMV$^{W122A}$-GFP replication and cell-to-cell movement

To determine whether TVBMVCPO$^{WT}$ (wild-type) or TVBMVCPO$^{W122A}$ could rescue the defective cell-to-cell movement of TVBMV$^{W122A}$, GFP, we infiltrated N. benthamiana leaves with a mixture of three Agrobacterium cultures harbouring pCamTVBMV$^{W122A}$-GFP, pCamTVBMVCPO$^{WT}$, or pCamTVBMVCPO$^{W122A}$, and pBinP19. The results showed that, by 132 hpi, N. benthamiana leaves coexpressing TVBMV$^{W122A}$, GFP, TVBMVCPO$^{WT}$, and P19 showed GFP fluorescence in multiple cells, indicating that TVBMVCPO$^{WT}$ rescued the cell-to-cell movement of TVBMV$^{W122A}$-GFP (Figure 6a,b). In contrast, the N. benthamiana leaves coexpressing TVBMV$^{W122A}$-GFP, TVBMVCPO$^{W122A}$, and P19 showed GFP fluorescence in single cells only, indicating that TVBMVCPO$^{W122A}$ could not rescue the cell-to-cell movement of TVBMV$^{W122A}$-GFP.

To determine whether TVBMVCPO$^{WT}$ and TVBMVCPO$^{W122A}$ could rescue the replication of TVBMV$^{W122A}$-GFP, we infiltrated N. benthamiana leaves with a mixture of Agrobacterium cultures harbouring pCamTVBMV$^{W122A}$-GFP, pCamTVBMVCPO$^{WT}$, or
pCamTVBMVCPW122A, and pBinP19, and analysed the harvested tissue samples through RT-qPCR at 60 hpai. The accumulation level of TVBMVW122A-GFP (−)RNA in the leaf tissues coexpressing TVBMVCPWT was partially rescued, whereas the accumulation level of TVBMVW122A-GFP (−)RNA in the leaf tissues coexpressing TVBMVCPW122A was not changed (Figure 6c), indicating that TVBMVCPWT could rescue the replication of TVBMVW122A-GFP.

We further found that TVBMVCPWT and TVBMVCPW122A could not rescue the replication and cell-to-cell movement of the replication-deficient mutant TVBMVCPSTOP-GFP (Figure 6), suggesting that efficient viral replication is essential for TVBMV cell-to-cell movement.

2.7 | The aromatic ring of W122 is crucial for CP stability, TVBMV replication, and movement

Several studies have shown that aromatic residues in proteins play essential roles in protein stability and function (Budyak et al., 2013; Butterfield et al., 2002; Chatterjee et al., 2019; Rege et al., 2018).

To determine whether the aromatic ring of the residue W122 was responsible for TVBMV cell-to-cell movement and CP stability, we mutated the codon for residue W122 in pCamTVBMV-GFP to the codons for the aromatic residue phenylalanine (F) or tyrosine (Y) to produce pCamTVBMVW122F-GFP (TVBMVW122F-GFP) and pCamTVBMVW122Y-GFP (TVBMVW122Y-GFP), and then individually infiltrated them into N. benthamiana leaves. By 7 dpai, both mutants TVBMVW122F-GFP and TVBMVW122Y-GFP caused systemic infection in N. benthamiana, although the GFP fluorescence from these two mutants was weaker than that of TVBMV-GFP under a UV light (Figure 7a). Western blot results revealed that the two mutants’ CP accumulated approximately 82% and 77%, respectively, of the TVBMV-GFP CP level (Figure 7b). Analysis using RT-qPCR showed that, at 60 hpai, the accumulation levels of viral RNA in the systemic leaves and (−)RNA in the TVBMVW122F-GFP and TVBMVW122Y-GFP-inoculated N. benthamiana leaf patches were approximately 60% of the TVBMV-GFP level (Figure 7c,d). We then mutated the W122 codon in pCamGFP-TVBMVCP to produce pCamGFP-TVBMVCPW122F (GFP-TVBMVCPW122F) and

FIGURE 5 Effect of W122 on CP subcellular localization. In this experiment, we coinfiltrated Nicotiana benthamiana leaves with mixed Agrobacterium cultures harbouring various combinations of constructs: (a) pCamGFP-TVBMVCP, pCamTVBMV, pCamP3N-PIPO, and pCamCI-DsRed; (b) pCamGFP-TVBMVCPW122A, pCamTVBMV, pCamP3N-PIPO, and pCamCI-DsRed. The infiltrated leaf tissues were collected at 48 hr posterior infiltration (hpai) and examined under a confocal microscope. Both GFP-TVBMVCP and GFP-TVBMVCPW122A were colocalized with CI-DsRed at the periphery of N. benthamiana leaf cells in the presence of TVBMV P3N-PIPO and TVBMV infection. White arrows indicate the points with both GFP-TVBMVCP and CI-DsRed or GFP-TVBMVCPW122A and CI-DsRed. Bars = 5 μm. Pictures were photographed at 60 hpai under a confocal microscope.
pCamGFP-TVBMVCP<sub>W122Y</sub> (GFP-TVBMVCP<sub>W122Y</sub>), and individually infiltrated them into <i>N. benthamiana</i> leaves. At 4 dpi, the GFP fluorescence intensity from GFP-TVBMVCP<sub>W122F</sub> and GFP-TVBMVCP<sub>W122Y</sub> reached approximately 60% of GFP-TVBMVCP, whereas that from GFP-TVBMVCP<sub>W122A</sub> was only 17% (Figure 7e,f). When the codon for residue A in pCamGFP-TVBMVCP<sub>W122A</sub> was changed back to the codon for residue W, the reverted mutant GFP-TVBMVCP<sub>W122A-W</sub> produced similar GFP fluorescence as GFP-TVBMVCP (Figure S6a,b). In this study, we also mutated the codon for W in pCamGFP-TVBMVCP to codons for non-aromatic residues R, asparagine (N), D, C, E, glutamine (Q), glycine (G), H, isoleucine (I), leucine (L), K, methionine (M), proline (P), S, threonine (T), or valine (V). After the infiltration of these mutant constructs to <i>N. benthamiana</i> leaves, none of the mutant fusion proteins produced visible GFP fluorescence in the leaf patches (Figure S6a,b). When the above mutations were introduced into pCamTVBMV-GFP, all the mutant viruses were confined to single cells (Figure S6c). Deletion of the W<sub>122</sub> codon from pCamGFP-TVBMVCP or pCamTVBMV-GFP reduced the accumulation level of the mutant GFP-TVBMVCP<sub>W122A</sub> and abolished the cell-to-cell movement of TVBMV<sub>W122del</sub>-GFP (Figure S6).

### 2.8 The corresponding residues of W<sub>122</sub> in watermelon mosaic virus CP or potato virus Y CP are also critical for viral cell-to-cell movement and CP accumulation

W<sub>133</sub> in watermelon mosaic virus (WMV) CP and residue W<sub>118</sub> in potato virus Y (PVY) CP are the corresponding residues of W<sub>122</sub> in TVBMV CP. To investigate whether W<sub>133</sub> and W<sub>118</sub> were also crucial for viral cell-to-cell movement, we deleted the codon for W<sub>133</sub> in pCBWMV-GFP and the codon for W<sub>118</sub> in pCamPVY-GFP to produce pCBWMV<sub>W133del</sub>-GFP and pCamPVY<sub>W118del</sub>-GFP, respectively. We then individually infiltrated pCBWMV-GFP, pCBWMV<sub>W133del</sub>-GFP, pCamPVY-GFP, and pCamPVY<sub>W118del</sub>-GFP into <i>N. benthamiana</i> leaves.
leaves. At 132 hpi, GFP fluorescence was observed in clusters of multiple cells infected with WMV-GFP or PVY-GFP. In contrast, GFP fluorescence from WMV\(_{W133del}\)-GFP or PVY\(_{W118del}\)-GFP was confined to single cells (Figure 8a,b), indicating that residues W\(_{133}\) and W\(_{118}\) were also crucial for viral cell-to-cell movement.

To investigate the roles of W\(_{133}\) and W\(_{118}\) in viral CP accumulation, we constructed pCamGFP-WMVCP, pCamGFP-WMVCP\(_{W133del}\), pCamGFP-PVYCP, and pCamGFP-PVYCP\(_{W118del}\), and then individually infiltrated them into N. benthamiana leaves. The results showed that, at 4 dpi, the GFP fluorescence from the two mutant proteins was significantly weaker than that from the two parental proteins (Figure 8c,d), indicating that residue W\(_{133}\) in WMV CP and residue W\(_{118}\) in PVY CP are crucial for CP accumulation.

### DISCUSSION

We studied the role of the conserved residue W\(_{122}\) in the TVBMV CP core domain in viral cell-to-cell movement and replication. Our results showed that mutation of W\(_{122}\) to various nonaromatic residues reduced viral replication and CP stability, leading to a defective viral cell-to-cell movement. Additionally, the aromatic ring of W\(_{122}\) was a key determinant of TVBMV CP stability, viral replication, and cell-to-cell movement.

CP is one of the potyviral proteins involved in viral cell-to-cell movement. It has been reported that a change in a single residue in the CP can affect potyviral cell-to-cell movement. For example, mutation of conserved residue S\(_{129}\), R\(_{161}\), or D\(_{205}\) (positions are numbered according to the TEV CP sequence) abolishes viral cell-to-cell movement (Dolja et al., 1994, 1995). In this study, the aromatic residue W\(_{122}\) in the CPs of 139 potyviruses was completely conserved (Figure S1). Mutation of W\(_{122}\) to various nonaromatic residues abolished TVBMV cell-to-cell movement (Figures 1e,f and S6c). Additionally, the deletion of W\(_{133}\) residue from WMV CP and the W\(_{118}\) residue from PVY CP abolished WMV and PVY cell-to-cell movement in N. benthamiana leaves (Figure 8a,b). Because W\(_{118}\) and W\(_{133}\) corresponded to W\(_{122}\) in TVBMV CP, we concluded that the conserved residue W\(_{122}\) was a key determinant of the potyviral cell-to-cell movement.

It is noteworthy that the three TEV movement-deficient mutants derived from S\(_{129}\), R\(_{161}\), and D\(_{205}\) do not produce virus particles (Dolja et al., 1994, 1995). The deletion of 65 residues from the C-terminus of
wheat streak mosaic virus (Tritimovirus, family Potyviridae) CP inhibits viral cell-to-cell movement but has no apparent effect on virus particle formation (Tatineni et al., 2014). We found that mutations of \( W^{122} \) to A, E, or K did not change TVBMV particle morphology, but inhibited viral cell-to-cell movement (Figures 1 and 4). Wei et al. (2010) reported that in the presence of turnip mosaic virus (TuMV) P3N-PIPO or during TuMV infection, TuMV CP and CI can accumulate adjacent to PD, which is a necessary step during potyviral cell-to-cell movement. In this study, we discovered that TVBMV CP and CI also accumulated adjacent to the PD in the presence of P3N-PIPO and TVBMV infection. Furthermore, the ability of TVBMV CP to target PD was not affected by the change in \( W^{122} \) to A (Figure 5). Therefore, we concluded that the role of \( W^{122} \) was in viral cell-to-cell movement, but not in viral RNA encapsidation.

Previous studies have shown that potyviral replication and cell-to-cell movement are coupled processes (Chai et al., 2020; Cui & Wang, 2016). Here, we found that substitution of conserved \( W^{122} \) to nonaromatic residues significantly inhibited, but did not abolish, the replication of mutant viruses (Figure 2c,d). Interestingly, the CP accumulation levels of these \( W^{122} \) virus mutants were similar to that of the defective virus mutant TVBMV\( ^{Nib_{GDD}} \)-GFP (Figure 2b). The abolished cell-to-cell movement of TVBMV\( ^{W^{122}A} \)-GFP could be rescued by coexpressing TVBMV\( ^{CPW} \) (Figure 6). In the same experiment, the RNA accumulation level of TVBMV\( ^{W^{122}A} \)-GFP was also increased. These results indicate that the reduced viral replication and extremely low CP accumulation might be both responsible for the defective cell-to-cell movement of \( W^{122} \) mutant viruses.

Potyviruses adopt a polyprotein expression strategy. The accumulation of individual viral proteins in the same tissues is assumed to be at the same level. The accumulating levels of (+)RNA and (-)RNA derived from \( W^{122} \) mutant viruses were much higher than those of the TVBMV\( ^{Nib_{GDD}} \)-GFP and TVBMV\( ^{CPW} \) mutant viruses (Figure 2c,d). The GFP accumulation levels of the three \( W^{122} \) mutant viruses were significantly higher than that of TVBMV\( ^{Nib_{GDD}} \), but not the accumulation levels of their CPs (Figure 2a,b). We considered that the mutations introduced to \( W^{122} \) destabilized the CPs. This conclusion was supported because substitution of A for \( W^{122} \) in the polyprotein N1a:HA-Nlb:GFP:CP did not change the accumulation levels of HA-Nlb and GFP, but reduced the accumulation level of mutant CP (Figure 3c).
Ubiquitin signalling and autophagy are two critical pathways that control protein degradation (Goldberg, 2012; Ožlmann et al., 2004, 2007). It has been reported that inhibition of proteasome activity using MG132 enhanced potato virus A CP accumulation (Löhmus et al., 2017), indicating that ubiquitin signalling can regulate virus CP degradation. The conserved helix in the C-terminal region of the NSs protein of the watermelon silver mottle virus (WSMoV) is critical for protein stability. However, the accumulation of WSMoV with mutations in the conserved helix is not affected by MG132 treatment (Huang et al., 2020). In this study, treatment of leaves with MG-132 or 3-MA, a specific inhibitor of autophagy (Seglen & Gordon, 1982), had no significant effect on the accumulation of GFP-TVBMVCP or mutant GFP-TVBMVCPW122A (Figure 5), suggesting that ubiquitin signalling and autophagy may not be the factors causing TVBMV CP instability.

The aromatic residues W, F, and Y are known to be important for protein stability and function. For example, the aromatic ring of Y598 in the WSMoV NSs protein has been shown to affect NSs stability and its RNA silencing suppression activity (Huang et al., 2015). Deletion of five residues, including an aromatic residue, from the readthrough protein of potato leafroll virus affects viral systemic infection and disease symptom induction (Xu et al., 2018). In this study, after mutation of W122 to nonaromatic residue A, E, or K, the mutant viruses produced destabilized CPs and exhibited reduced viral replication and defective viral cell-to-cell movement (Figures 1 and 2). Substitutions of other nonaromatic residues for W122 produced the same results (Figure S6). In contrast, changing residue W122 to aromatic residues F or Y yielded two mutant viruses capable of replicating to c.60% of TVBMV-GFP in the infiltrated leaves and c.80% of TVBMV CP in the systemically infected leaves (Figure 7d). Because similar results were obtained for WMV and PVY CPs (Figure 8), we concluded that the aromatic residue W122 was a determinant of potyviral CP stability, replication, and movement. Aromatic rings can interact with each other to stabilize proteins. In a recent report, aromatic ring interaction between Y52 and F64 in the small ubiquitin-like modifier (SUMO) is vital to SUMO stability and its SUMOylation activity (Chatterjee et al., 2019). We speculate that the aromatic ring of W122 also interacts with other residues to stabilize CP, leading to successful cell-to-cell movement.

The aromatic ring of residue W122 is a critical factor for potyviral replication and CP stability. As a result, mutation of W122 to a nonaromatic residue abolishes potyviral cell-to-cell movement. The findings presented here increase our understanding of the underlying mechanism controlling potyviral cell-to-cell movement. Further studies are necessary to elucidate the roles of CP secondary or higher structures during potyvirus infection in plants.

4 | EXPERIMENTAL PROCEDURES

4.1 | Amino acid sequence alignments

CP sequences of 139 potyviruses were downloaded from a reference sequence database at the National Center for Biotechnology Information (O’Leary et al., 2016). Multiple sequence alignments were performed using the ClustalW program in BioEdit v. 7.2.6, using the default parameters (Hall, 1999). The resulting FASTA file was further processed using the online application WebLogo (http://weblogo.berkeley.edu) to generate sequence logos as previously described (Crooks, 2004).

4.2 | Plasmid construction and site-directed mutagenesis

The gfp gene-containing infectious clones pCamTVBMV-GFP (accession number: JQ407082), pCBWMV-GFP, pCamPVY-GFP, and the infectious clone pCamTVBMV without gfp gene were constructed in our laboratory (Gao et al., 2012; Geng et al., 2015). The coding sequences of TVBMV CP, WMV CP, and PVY CP were PCR-amplified from the above infectious clones and individually inserted into the expression vector pCam35S::GFP to produce pCamGFP-TVBMVCP, pCamGFP-WMVCP, and pCamGFP-PVYCP, respectively. The TVBMV CI coding region was PCR-amplified and cloned into a different expression vector pCam35S::DsRed to produce pCamCI-DsRed. The cloning regions of TVBMV P3N-PIPO, CP, and NlaPro:Nlb:GFP:CP were PCR-amplified and cloned individually into the pCam35S vector to produce pCamTVBMV-CPW1, pCamP3N-PIPO, and pCamNlaPro:Nlb:GFP:CP, respectively. An HA-tag encoding sequence was inserted between the first and second codons of the Nlb open reading frame (ORF) in the pCamNlaPro:Nlb:GFP:CP clone to produce pCamNlaPro:HA-Nlb:GFP:CP. For expression of TVBMV CP in E. coli cells, the CP sequence was cloned into vector pEHISTEV to produce pEHISTEV-TVBMVCP. To generate a replication-deficient TVBMV mutant, we deleted the codons for the conserved GDD motif in the Nlb ORF in pCamTVBMV-GFP to produce pCamTVBMVΔGDD-GFP as described (Geng et al., 2017). A stop codon was inserted immediately after the second codon in the CP ORF in pCamTVBMV-GFP to produce pCamTVBMVCPSTOP-GFP. Substitutions of the codons for alanine (A), glutamic acid (E), or lysine (K) for that of W122 in pCamTVBMV-GFP were performed using site-directed mutagenesis as previously described (Liu & Naismith, 2008) to produce pCamTVBMVW122A-GFP, pCamTVBMVW122E-GFP, and pCamTVBMVW122K-GFP, respectively. The codon for W133 in pCBWMV-GFP and the codon for W118 in pCamPVY-GFP were deleted to produce pCBWMVΔW133del-GFP and pCamPVYΔW118del-GFP, respectively. All plasmids were sequenced before use. Primers used in this study are listed in Table S1. The names and products of various constructs used in this study are listed in Table S2.

4.3 | Plant growth, virus inoculation, and protein transient expression

N. benthamiana plants were grown in a greenhouse set at 25 °C and a 16/8 hr (light/dark) photoperiod. Plasmids were individually
transformed into Agrobacterium tumefaciens GV3101 cells using a freeze-thaw method. Agrobacterium cultures harbouring different plasmids were cultured overnight in Luria–Bertani liquid medium supplemented with appropriate antibiotics at 28 °C with 220 rpm shaking. The cultures were pelleted through centrifugation at 8,000 × g for 2 min and individually resuspended in an induction buffer containing 10 mM MgCl₂, 10 mM MES, and 150 μM acetosyringone. To determine viral accumulation in the infiltrated leaf patches and systemic movement in the plants, the agrobacterium cultures were diluted to OD₆₅₀ = 0.2. To determine viral cell-to-cell movement, the agrobacterium cultures were diluted to OD₆₅₀ = 0.0001.

For transient expression assays, individual agrobacterium cultures were diluted to OD₆₅₀ = 0.4 and then mixed with an agrobacterium culture (1:1 vol/vol) harbouring pBinP19, a vector expressing tobacco bushy stunt virus P19 protein. For cell-to-cell movement trans-complementation assays, the agrobacterium cultures carrying pCamTVBMVCPSTOP-GFP or pCamTVBMVpW122A-GFP were diluted to OD₆₅₀ = 0.0003 and the cultures carrying pCamTVBMVCWPWT or pCamTVBMVCWPW122A were diluted to OD₆₅₀ = 0.6. These cultures were individually mixed with an equal volume of culture carrying pBinP19 (OD₆₅₀ = 0.6). For viral replication trans-complementation assays, the agrobacterium cultures carrying pCamTVBMVCSTOP-GFP or pCamTVBMVCWPW122A-GFP, pCamTVBMVCWPWT or pCamTVBMVCWPW122A, and pBinP19 were diluted to OD₆₅₀ = 0.6 and mixed at a ratio of 1:1:1. After 3 hr incubation at 25 °C, the cultures were individually infiltrated into leaves of 4–6-week-old N. benthamiana plants with 1-ml needleless syringes. The infiltrated plants were grown in a greenhouse till use. For protein degradation assays, a 1% dimethyl sulphoxide (DMSO) (control) solution or 50 μM MG132 (Selleck Chemicals) were individually infiltrated into leaves of 4–6-week-old N. benthamiana ef1a plants and used for total RNA extraction. The collected leaf tissues were individually homogenized (1:8, wt/wt) and then treated with a gDNA wipe enzyme (Vazyme) to remove plant genomic DNA. For RT-qPCR analysis of viral RNA accumulation, 500 ng total RNA (per sample) was reverse transcribed using primers qTVBMVCpF and EF1A-R, and was used as an internal control.

4.4 | RNA extraction and RT-qPCR

The infiltrated leaf tissues or the systemic leaves were collected from the assayed N. benthamiana plants and used for total RNA extraction using TransZol reagent (TransGen Biotech). The resulting total RNA samples were treated with a gDNA wipe enzyme (Vazyme) to remove plant genomic DNA. For RT-qPCR analysis of viral RNA accumulation, 500 ng total RNA (per sample) was reverse transcribed using a HiScript II Q RT SuperMix kit supplemented with random primers (Vazyme). To detect viral plus- and minus-strand RNA accumulation in the infiltrated leaf tissues, 500 ng total RNA (per sample) was reverse transcribed using primers qTVBMVCp-R and EF1A-R or primers qTVBMVCp-F and EF1A-R, and the HiScript II Q RT select SuperMix. Quantitative PCR was conducted using a ChamQ SYBR qPCR Master Mix (Vazyme) on a thermocycler (LC96; Roche). TVBMVCp-specific qPCR primers are listed in Table S1. The expression of N. benthamiana ef1a was determined using primer EF1A-F and EF1A-R, and was used as an internal control.

4.5 | Western blot assay

Systemically infected leaves or the infiltrated leaf tissues were collected from the assayed N. benthamiana plants and homogenized individually in a protein extraction buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 5% sucrose, and 1 mM phenylmethylsulfonyl fluoride) at a ratio of 1:2 (wt/vol) using a tissue grinder (Jingxin). The leaf extracts were denatured at 95 °C for 5 min, incubated on ice for 5 min, and then centrifuged at 12,000 × g for 5 min. The supernatant was collected from each sample, and the proteins in each sample were separated in SDS-polyacrylamide gels through electrophoresis and then blotted onto nitrocellulose membranes. Polyclonal antibodies specific for TVBMV CP or GFP were prepared in our laboratory (Ji et al., 2018; Lan et al., 2007) and were all used at a 1:1,000 dilution. A horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich) was used as the secondary antibody diluted at 1:50,000 (vol/vol). After the addition of the SuperSignal West Dura extended duration substrate solution (Thermo Fisher Scientific), the detection signal was visualized using a chemiluminescent imaging and analysis system (Sage).

4.6 | ELISA

The collected plant tissues were individually homogenized (1:8, wt/wt) in a coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6). Aliquots (100 µl) of leaf crude extracts were added into individual wells on a 96-well microtiter plate and incubated overnight at 4 °C. After four rinses with a phosphate-buffered saline with Tween-20 (PBS-T; 80 mM Na₂HPO₄, 1.5 M NaCl, 20 mM KH₂PO₄, 30 mM KCl, 0.5% Tween-20, pH 7.4), the GFP or the TVBMVCp antibody solution was added into each well, and the plate was incubated at 37 °C for 4 hr. After four rinses with the PBS-T an alkaline phosphatase-conjugated goat anti-rabbit IgG solution was added to the wells followed by 4 hr incubation at 37 °C. After the addition of a p-nitrophenyl phosphate substrate solution (Sigma, 0.25 mg/ml), the absorbance value (A₄₀₅) of each well was measured using a microplate reader (BioTek Synergy Mx).

4.7 | Confocal microscopy and fluorescence intensity measurement

To monitor viral intercellular movement and protein subcellular localization in N. benthamiana leaves, the agrobacterium-infiltrated leaf patches were collected and examined under a laser confocal microscope (Carl Zeiss). For GFP fluorescence observation, the excitation and emission wavelengths were set at 488 and 520–540 nm, respectively. For DsRed fluorescence observation, the excitation and emission wavelengths were set at 561 and 590–630 nm, respectively. The captured images were processed using the ZEN 2.1 (Carl Zeiss). Agrobacterium-infiltrated N. benthamiana leaves were photographed under UV light (365 nm) from
a hand-held UV lamp (LUYOR) using a digital camera (Canon 80D). To determine GFP fluorescence intensity in the infiltrated leaf tissues, leaf discs (5 mm in diameter) were sampled from the infiltrated N. benthamiana leaf patches with a cork borer and individually placed in wells of a 96-well microtitre plate. GFP fluorescence from each well was determined using a microplate reader (BioTek). The excitation wavelength was 485/10 nm and the emission wavelength was 535/10 nm.

4.8 | Virus particle purification

Virus particles were purified from the infiltrated N. benthamiana leaves. At 5 dpi, 15 g of tissues were harvested from the infiltrated leaves, ground in liquid nitrogen, and then homogenized in 30 ml of 0.2 M phosphate buffer at pH 8 and supplemented with 0.15% β-mercaptoethanol and 0.01 M EDTA. The crude extracts were centrifuged at 8,000 × g for 20 min. The supernatant was filtered through four layers of cheesecloth and stirred at 4 °C for 3 hr after adding 1% Triton X-100, 40 g/L polyethylene glycol 6,000, and 0.2 M NaCl. Virus particles were precipitated by centrifugation at 8,000 × g for 20 min, and the pellets were resuspended overnight at 4 °C in a 0.2 M phosphate buffer at pH 8 and containing 1% Triton X-100. Insoluble materials were removed by centrifugation at 8,000 × g for 20 min, and virus particles in the supernatant were pelleted through 1 hr ultracentrifugation at 100,000 × g at 4 °C using an ultracentrifuge CP100WX (Hitachi). The pellets were individually resuspended overnight in a 0.05 M phosphate buffer at pH 8 and at 4 °C. The insoluble materials were removed again by 20 min centrifugation at 8,000 × g. Small amounts of supernatant from each sample were negatively stained with 2% uranyl acetate, loaded onto 230-mesh carbon-coated copper grids, and then examined under a JEM-1200Ex transmission electron microscope (Jeol) for virus particle morphology.

4.9 | Protein expression

Plasmids for expressing the wild-type or mutant CPs were individually transformed into E. coli Rosetta cells, and the transformed cells were cultured until OD600 = 0.6. The cultures were induced with a 0.1 mM isopropyl-β-D-thiogalactopyranoside solution and incubated at 16 °C for 12 hr. The cultures were pelleted by centrifugation at 8,000 × g for 5 min, and the pellets were individually resuspended with a lysis buffer (50 mM NaHPO4, 300 M NaCl, pH 8.0) followed by 5 min of lysis through sonication on ice. The lysed cells were pelleted by centrifugation at 10,000 × g for 5 min. Supernatants of each sample were transferred into a new clean tube and used as soluble proteins for SDS-PAGE and western blot assays.

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DATA AVAILABILITY STATEMENT

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

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**SUPPORTING INFORMATION**
Additional supporting information may be found online in the Supporting Information section.

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