Residues R¹⁹² and K²²⁵ in RNA-Binding Pocket of Tobacco Vein Banding Mosaic Virus CP Control Virus Cell-to-Cell Movement and Replication

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Potyviruses move to neighboring cells in the form of virus particles or a coat protein (CP)-containing ribonucleoprotein complex. However, the precise roles of RNA-binding residues in potyviral CP in viral cell-to-cell movement remain to be elucidated. In this study, we predicted the three-dimensional model of tobacco vein banding mosaic virus (TVBMV)-encoded CP and found nine residues presumably located in the CP RNA-binding pocket. Substitutions of the two basic residues at positions 192 and 225 (R¹⁹² and K²²⁵) with either alanine, cysteine, or glutamic acid abolished TVBMV cell-to-cell and systemic movement in *Nicotiana benthamiana* plants. These substitutions also reduced the replication of the mutant viruses. Results from the electrophoretic mobility shift assay showed that the RNA-binding activity of mutant CPs derived from R¹⁹² or K²²⁵ substitutions was significantly lower than that of wild-type CP. Analysis of purified virus particles showed that mutant viruses with R¹⁹² or K²²⁵ substitutions formed RNA-free virus-like particles. Mutations of R¹⁹² and K²²⁵ did not change the CP plasmodesmata localization. The wild-type TVBMV CP could rescue the deficient cell-to-cell movement of mutant viruses. Moreover, deletion of any of the other seven residues also abolished TVBMV cell-to-cell movement and reduced the CP RNA-binding activity. The corresponding nine residues in watermelon mosaic virus CP were also found to play essential roles in virus cell-to-cell movement. In conclusion, residues R¹⁹² and K²²⁵ in the CP RNA-binding pocket are critical for viral RNA binding and affect both virus replication and cell-to-cell movement.

Keywords: cell-to-cell movement, coat protein, *Potyvirus*, RNA-binding activity, *Tobacco vein banding mosaic virus*, virus movement

Cell-to-cell movement is one of the critical steps for plant viruses to establish systemic infection (Ritzenthaler 2011; Schoelz et al. 2011). Virus-encoded proteins cooperatively assist plant viral RNA to enter neighboring cells via plasmodesmata (PD). For example, the movement protein and the 126/183-kDa replicase protein are all involved in the tobacco mosaic virus (TMV, genus *Tobamovirus*) cell-to-cell movement (Hirashima and Watanabe 2001, 2003; Yuan et al. 2016). The triple gene block proteins and the coat protein (CP) are needed for potato virus X (PVX, genus *Potexvirus*) cell-to-cell movement (Tilsner et al. 2013). Turnip mosaic virus (TuMV, genus *Potyvirus*) requires at least five viral proteins, including the third protein (P3) (Chai et al. 2020); frame-shift protein, including the N terminus of P3 and residues encoded by pretty interesting potyvirus open reading frame (P3N-PIPO) (Cheng et al. 2017; Geng et al. 2015; Olsper et al. 2015; Rodamilans et al. 2015; Wei et al. 2010); cylindrical inclusion (CI) (Carrington et al. 1998; Deng et al. 2015; Movahed et al. 2017; Roberts et al. 1998; Rodríguez-Cerezo et al. 1997); the second 6-kDa protein (6K2) (Grangeon et al. 2013; Jiang et al. 2015); and CP (Dolja et al. 1994, 1995), for its cell-to-cell movement.

The N-terminal, core, and C-terminal regions of potyvirus CPs play critical roles in virus cell-to-cell movement. Maintenance of the net charge, not the primary sequence, of the N-terminal region of CP is required for cell-to-cell movement of zucchini yellow mosaic virus (ZYMV) (Kimalov et al. 2004). Mutation of the histidine at position 246 (H²⁴⁶) or aspartic acid at position 250 (D²⁵⁰) of the C-terminal region of CP inhibits the intercellular movement of soybean mosaic virus (SMV) (Kimalov et al. 2004; Seo et al. 2013). Mutations of the phosphorylation site T²⁴³ in the CP core region abolish the replication and cell-to-cell movement of potato virus A (PVA) (Ivanov et al. 2003; Lohmus et al. 2017). Residues serine (S¹²²), arginine (R¹⁵⁴), and aspartic acid (D¹⁹⁸) of the CP core region are also known as the determinants for the cell-to-cell movement of tobacco etch virus (TEV) (Dolja et al. 1994, 1995). The ring of aromatic residue (W¹²²) is critical for CP stability, virus replication, and cell-to-cell movement of tobacco vein banding mosaic virus (TVBMV), potato virus Y (PVY), and watermelon mosaic virus (WMV) (Yan et al. 2021). The three-dimensional (3D) structures of WMV, PVY, and TuMV CPs suggest that residues

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S, R, and D are located in the RNA-binding pocket of CP and are probably involved in RNA binding (Cuesta et al. 2019; Kežar et al. 2019; Zamora et al. 2017). However, the precise RNA-binding residues in potyvirus CPs and the roles of these residues in virus cell-to-cell movement remain to be elucidated.

In this study, we have determined that the residues located in the RNA-binding pocket of potyvirus CPs are involved in the viral cell-to-cell movement by maintaining the RNA-binding activity of CP.

RESULTS

Nine amino acid residues in the core region of TVBMV CP were predicted to be in the RNA-binding pocket.

To determine the RNA-binding sites of TVBMV CP, we predicted the 3D model of TVBMV CP using the I-TASSER server. We then aligned the sequences that were used to create the 3D model of the WMV CP-RNA complex (Zamora et al. 2017) with that of TVBMV CP (RNA modeled as a polyU), and found that these two CPs shared the same 3D model structures (Supplementary Fig. S1). The nucleotide-labeled U4 was located in the CP RNA-binding pocket (Fig. 1A). Further analysis of the TVBMV CP-RNA model showed that nine residues—S, proline (P), R, tyrosine (Y), R, D, lysine (K), alanine (A), and valine (V) at positions 129 (S^{129}), 130 (P^{130}), 161 (R^{161}), 188 (Y^{188}), 192 (R^{192}), 205 (D^{205}), 225 (K^{225}), 228 (A^{228}), and 229 (V^{229}), respectively—were within 4 Å distance from the nucleotide U4 (Fig. 1A), and were predicted to be in the RNA-binding pocket. These nine residues were all in the core region of TVBMV CP (Fig. 1B).

Mutations of R^{192} and K^{225} abolished TVBMV systemic and cell-to-cell movement.

To investigate the roles of the residues within the RNA-binding pocket of CP in TVBMV infection, we selected two positively charged residues, R^{192} and K^{225} , for mutagenesis. The codons for R^{192} and K^{225} in infectious clone pCamTVBMV-green fluorescent protein (GFP) (Gao et al. 2012; Geng et al. 2015) (the derived virus was named TVBMV-GFP) were substituted with those for residues with different properties, including A, cysteine (C), and glutamic acid (E), to produce six mutant plasmids: pCamTVBMV R^{192A} -GFP (TVBMV R^{192A} -GFP), pCamTVBMV R^{192C} -GFP (TVBMV R^{192C} -GFP), pCamTVBMV R^{192E} -GFP (TVBMV R^{192E} -GFP), pCamTVBMV K^{225A} -GFP (TVBMV K^{225A} -GFP), pCamTVBMV K^{225C} -GFP (TVBMV K^{225C} -GFP), and pCamTVBMV K^{225E} -GFP (TVBMV K^{225E} -GFP), respectively. Agrobacterial cultures carrying various mutant plasmids were adjusted to an optical density at 600 nm (OD_{600}) = 0.2 and individually infiltrated into *Nicotiana benthamiana* leaves. At 7 days postagroinfiltration (dpi), the systemic and infiltrated leaves of *N. benthamiana* plants inoculated with TVBMV-GFP showed distinct GFP fluorescence under UV illumination. However, only the infiltrated leaves of the *N. benthamiana* plants inoculated with mutant viruses displayed GFP fluorescence (Fig. 2A). Virus accumulation in the systemic leaves was then detected by enzyme-linked immunosorbent assay (ELISA) and the results showed that viral CP had accumulated in the systemic leaves of the plants inoculated with TVBMV-GFP but not in that of the mutant virus-inoculated plants (Fig. 2B). We further examined the cell-to-cell

Fig. 1. Identification of the amino acid residues located in the coat protein (CP) RNA-binding pocket. **A**, A three-dimensional model of the tobacco vein banding mosaic potyvirus (TVBMV) CP-RNA complex. TVBMV CP is shown as a white ribbon. The polyU RNA is indicated in green, and the nucleotide U4 located in the RNA-binding pocket is shown in magenta. Residues S^{129} , P^{130} , R^{161} , Y^{188} , R^{192} , D^{205} , K^{225} , A^{228} , and V^{229} within 4 Å to the nucleotide U4 are indicated in blue, cyan, orange, tint, and yellow. **B**, Schematic diagram showing the genome organization of the infectious clone pCamTVBMV-green fluorescent protein (GFP) (accession number JQ407082). The N- and C-terminal domains (N-ter and C-ter) of TVBMV CP are shown in dark gray, and the core region (Core) is shown in black. UTR = untranslated region and P3N-PIPO = N terminus of P3 and residues encoded by pretty interesting potyvirus open reading frame. Relative positions of S^{129} , P^{130} , R^{161} , Y^{188} , R^{192} , D^{205} , K^{225} , A^{228} , and V^{229} are indicated by the red arrowhead.

movement of these mutant viruses. Agrobacterial cultures harboring pCamTVBMV-GFP or one of its mutant plasmids were diluted to $OD_{600} = 0.0001$ and individually infiltrated into *N. benthamiana* leaves. At 5 dpai, GFP fluorescence appeared in many cells in each locus in the *N. benthamiana* leaves inoculated with TVBMV-GFP; in contrast, GFP fluorescence produced by mutant viruses was confined in single cells (Fig. 2C and D). These results indicated that the mutations of R¹⁹² or K²²⁵ abolished TVBMV cell-to-cell and systemic movement in its host plants.

Mutations of R¹⁹² and K²²⁵ also reduced TVBMV replication.

To explore whether the mutations of R¹⁹² and K²²⁵ could affect TVBMV replication, we agroinfiltrated *N. benthamiana* leaves with pCamTVBMV-GFP or individual R¹⁹²- or K²²⁵-derived mutant plasmids. Plants agroinfiltrated with the replication-deficient mutants pCamTVBMV^{NibΔGDD}-GFP (TVBMV^{NibΔGDD}-GFP) and pCamTVBMV^{CPSTOP}-GFP (TVBMV^{CPSTOP}-GFP) were used as controls (Geng et al. 2017; Yan et al. 2021). Agrobacterial cultures were adjusted to $OD_{600} = 0.2$. The accumulations of GFP and CP in the agroinfiltrated leaf patches were separately analyzed by ELISA. At 60 h postagroinfiltration (hpa), mutants TVBMV^{R192C}-GFP, TVBMV^{K225A}-GFP, TVBMV^{K225C}-GFP, and TVBMV^{K225E}-GFP accumulated significantly less GFP and CP than TVBMV-GFP, and mutants TVBMV^{R192A}-GFP and

TVBMV^{R192E}-GFP accumulated even less GFP and CP. In this study, mutants TVBMV^{NibΔGDD}-GFP and TVBMV^{CPSTOP}-GFP accumulated the lowest levels of GFP and CP (Fig. 3A and B). Similar GFP and CP accumulation levels were observed for these mutants at 108 hpa (Supplementary Fig. S2). The accumulation level of TVBMV minus-strand (–)RNA in various agroinfiltrated leaf samples was also analyzed using quantitative reverse-transcription PCR (qRT-PCR). The results showed that the relative accumulation levels of mutant viruses (–)RNA were significantly reduced (Fig. 3C), indicating that the replications of the R¹⁹²- and K²²⁵-derived mutants were significantly decreased.

Mutations of R¹⁹² and K²²⁵ also reduced CP RNA-binding activity.

To test whether the mutations of R¹⁹² or K²²⁵ could affect the RNA-binding activity of CP, we conducted an electrophoretic mobility shift assay (EMSA). To express and purify CP in vitro, we constructed expression vector pEHISTEV-TVBMVCP (the expressed protein was named His-TVBMVCP) and mutant vectors pEHISTEV-TVBMVCP^{R192A} (His-TVBMVCP^{R192A}) and pEHISTEV-TVBMVCP^{K225A} (His-TVBMVCP^{K225A}), respectively. These vectors were individually transformed into *Escherichia coli* Rosetta cells. Because potyvirus CP binds RNA in a nonspecific manner (Besong-Ndika et al. 2015), we selected a fragment (5′-721GGA AAU GUC AGC ACG AAG GAG GAA AAC ACA⁷⁵⁰-3′) from the TVBMV CP coding region as an

Fig. 2. Effects of residues R¹⁹² and K²²⁵ mutations on tobacco vein banding mosaic potyvirus (TVBMV) systemic and cell-to-cell movement in *Nicotiana benthamiana* plants. **A**, Green fluorescence in *N. benthamiana* plants inoculated with TVBMV-green fluorescent protein (GFP) or its mutants derived from R¹⁹² and K²²⁵ mutations at 7 days postagroinfiltration (dpai). Pictures were captured under UV light. **B**, Accumulation of coat protein (CP) in systemic leaves of the *N. benthamiana* plants infiltrated with TVBMV-GFP or its mutants was determined by enzyme-linked immunosorbent assay at 7 dpai. The value of each column represents the means of three biological replicates and bars indicate the positive and negative standard deviations ($\pm$ SD). **C**, Cell-to-cell movement of TVBMV-GFP and its mutants derived from R¹⁹² or K²²⁵ mutation. GFP fluorescence in *N. benthamiana* leaf cells infected with TVBMV-GFP or its mutants was captured under a confocal microscope at 5 dpai. **D**, Number of cells showing GFP fluorescence per infection locus caused by TVBMV-GFP mutants at 5 dpai. The number of cells showing GFP fluorescence in each TVBMV-GFP infection locus could not be precisely determined and was not shown. The value of each column represents the means from 30 infection loci and bars indicate $\pm$ SD. Statistical differences were determined by the Duncan's multiple range test ($P < 0.05$). The same lowercase letter indicated no significant difference between the treatments.

Fig. 3. Effects of R^{192} and K^{225} mutations on tobacco vein banding mosaic potyvirus (TVBMV) replication. **A**, Green fluorescent protein (GFP) and **B**, TVBMV coat protein (CP) accumulating levels in the *Nicotiana benthamiana* leaf patches infiltrated with TVBMV-GFP and its mutants derived from the R^{192} and K^{225} mutations were determined by enzyme-linked immunosorbent assay at 60 h postagroinfiltration (hpa). Replication-deficient mutants TVBMV^{N1bΔGDD}-GFP and TVBMV^{CPSTOP}-GFP were used as controls. **C**, Quantitative reverse-transcription PCR analyses of relative accumulations of TVBMV (-)RNA in the *N. benthamiana* leaf patches infiltrated with TVBMV-GFP or its mutants at 60 hpa. The expression of gene *EF1α* was used as an internal control. The value of each column represents the means of three biological replicates and bars indicate the positive and negative standard deviations. Statistical differences were determined by the Duncan's multiple range test ($P < 0.05$). The same lowercase letter indicated no significant difference between the treatments.

RNA probe and labeled it with hexafluorescein (HEX). After incubation of this probe (8 pmol) with different amounts (0, 0.6, 1.2, or 2.4 μ M) of His-TVBMVCP or one of its mutants followed by electrophoresis, the His-TVBMVCP and RNA probe reaction gave a distinctive band in the gel; in contrast, the complex of His-TVBMVCP^{R192A} or His-TVBMVCP^{R192A} and RNA probe did not show distinct accumulation. The reaction containing glutathione S-transferase and the RNA probe was used as a negative control (Fig. 4). These results suggested that R^{192} and K^{225} were critical for the RNA-binding activity of CP.

Mutations of R^{192} and K^{225} did not affect CP PD localization.

PD localization is critical for the potyvirus cell-to-cell movement (Wei et al. 2010). To investigate the effects of mutations of R^{192} and K^{225} on CP subcellular localization, we coinfiltrated *N. benthamiana* leaves with mixed agrobacterial cultures ($OD_{600} = 1.0$) harboring five plasmids: pCamGFP-TVBMVCP, pCamTVBMV, pCamCI-DsRed, pCamP3N-PIPO, and pBinP19; or pCamGFP-TVBMVCP^{R192A}, pCamTVBMV^{R192A}, pCamCI-DsRed, pCamP3N-PIPO, and pBinP19; or pCamGFP-TVBMVCP^{K225A}, pCamTVBMV^{K225A}, pCamCI-DsRed, pCamP3N-PIPO, and pBinP19. All agrobacterial cultures were mixed at equal ratios. Agrobacterial culture harboring pBinP19 was coinfiltrated to increase the protein expression levels (Himber et al. 2003). At 48 hpa, GFP-TVBMV, GFP-TVBMVCP^{R192A}, or GFP-TVBMVCP^{K225A} all colocalized with CI-DsRed in the cell periphery (Fig. 5). CI-DsRed was previously reported to localize in the PDs in the presence of TVBMV P3N-PIPO (Geng et al. 2015). These results indicated that mutations of R^{192} or K^{225} did not change the subcellular localization of TVBMV CP.

Mutant viruses derived from R^{192} or K^{225} mutation formed RNA-free virus-like particles.

To determine whether mutations of R^{192} and K^{225} could affect virus particle formation, we individually agroinfiltrated pCamTVBMV-GFP, pCamTVBMV^{R192A}-GFP, and pCamTVBMV^{K225A}-GFP into *N. benthamiana* leaves. Agrobacterial cultures were adjusted to $OD_{600} = 0.2$. At 5 dpai, the infiltrated leaves were collected for virus particle purification. The obtained virus particles were examined under a transmission electron microscope. The results showed that TVBMV-GFP, TVBMV^{R192A}-GFP, and TVBMV^{K225A}-GFP all produced virus-like particles (VLPs) (Supplementary Fig. S3A), except that the numbers of VLPs produced by TVBMV^{R192A}-GFP and TVBMV^{K225A}-GFP were significantly less than that produced by TVBMV-GFP (Supplementary Fig. S3B). We then treated these VLPs with a genomic DNA (gDNA) wiper Mix reagent and RNase A to remove contaminative DNA and RNA before extracting VLP-encapsulated RNA using Trizol reagent. The resulting products were analyzed by RT-PCR. The results showed that TVBMV RNA was detected in the product obtained from TVBMV-GFP VLPs but not in the products obtained from TVBMV^{R192A}-GFP and TVBMV^{K225A}-GFP VLPs (Supplementary Fig. S3C), indicating that the TVBMV^{R192A}-GFP and TVBMV^{K225A}-GFP VLPs were viral RNA free.

Wild-type TVBMV CP could rescue the defective cell-to-cell movement of R^{192} and K^{225} mutants.

To determine whether the defective cell-to-cell movement of R^{192} and K^{225} mutant viruses could be rescued, we constructed plasmids pCBTVBMV^{R192A}-GFP/mCherry (TVBMV^{R192A}-GFP/mCherry) and pCBTVBMV^{K225A}-GFP/mCherry (TVBMV^{K225A}-GFP/mCherry) (Fig. 6A). After agroinfiltrating these plasmids, individually, into *N. benthamiana* leaves, the initially infected *N. benthamiana* cells showed both green and red

fluorescence. After the progeny viruses had moved into the neighboring cells, they produced only green fluorescence. In this study, we also constructed pCamMyc-TVBMVCP^{WT} (Myc-TVBMVCP^{WT}), pCamMyc-TVBMVCP^{R192A} (Myc-TVBMVCP^{R192A}), and pCamMyc-TVBMVCP^{K225A} (Myc-TVBMVCP^{K225A}) (Fig. 6A) and used them to express TVBMV with Myc-tagged wild-type CP, or TVBMV with the R¹⁹² and K²²⁵ mutant CP in plants. *N. benthamiana* leaves were coinfiltrated with mixed agrobacterial cultures harboring pCBTVBMV^{R192A}-GFP/mCherry and pCamMyc-TVBMVCP^{R192A}, pCBTVBMV^{R192A}-GFP/mCherry and pCamMyc-TVBMVCP^{WT}, pCBTVBMV^{K225A}-GFP/mCherry and pCamMyc-TVBMVCP^{K225A}, or pCBTVBMV^{K225A}-GFP/mCherry and pCamMyc-TVBMVCP^{WT}. In this assay, the agrobacterial cultures harboring pCBTVBMV^{R192A}-GFP/mCherry or pCBTVBMV^{K225A}-GFP/mCherry were used at OD₆₀₀ = 0.0003, while the cultures harboring other plasmids were used at OD₆₀₀ = 0.6. The three cultures in each treatment were mixed at equal ratios. At 5 dpai, green fluorescence was confined to the single *N. benthamiana* leaf cells coexpressing TVBMV^{R192A}-GFP/mCherry and Myc-TVBMVCP^{R192A}, or TVBMV^{K225A}-GFP/mCherry and Myc-TVBMVCP^{K225A} (Fig. 6B and C). In contrast, green fluorescence was observed in approximately 15 *N. benthamiana* leaf cells per locus coexpressing TVBMV^{R192A}-GFP/mCherry or TVBMV^{K225A}-GFP/mCherry with Myc-TVBMVCP^{WT} (Fig. 6B and C). The expressions of Myc-TVBMVCP^{WT}, Myc-TVBMVCP^{R192A}, and Myc-TVBMVCP^{K225A} in the infiltrated leaves were confirmed by a Western blotting assay (Fig. 6D). These results indicated that the defective cell-to-cell movement of the R¹⁹² and K²²⁵ mutant viruses was rescued by the transexpression of wild-type TVBMV CP.

Seven other residues in the RNA-binding pocket could also affect TVBMV cell-to-cell movement and the CP RNA-binding activity.

We further analyzed the roles of seven other residues (S¹²⁹, P¹³⁰, R¹⁶¹, Y¹⁸⁸, D²⁰⁵, A²²⁸, and V²²⁹) inside the CP RNA-binding pocket in TVBMV cell-to-cell movement by deleting the codons for these residues in the pCamTVBMV-GFP vector to obtain pCamTVBMV^{S129del}-GFP, pCamTVBMV^{P130del}-GFP, pCamTVBMV^{R161del}-GFP, pCamTVBMV^{Y188del}-GFP, pCamTVBMV^{D205del}-GFP, pCamTVBMV^{A228del}-GFP, and

pCamTVBMV^{V229del}-GFP, respectively. Agrobacterial cultures (OD₆₀₀ = 0.0001) harboring individual mutant plasmids or pCamTVBMV-GFP were infiltrated into *N. benthamiana* leaves. At 5 dpai, GFP fluorescence was observed in many cells per locus in the *N. benthamiana* leaves infected with TVBMV-GFP. In contrast, GFP fluorescence produced by mutant viruses was localized in single *N. benthamiana* cells (Fig. 7A and B).

To determine whether mutations of these seven residues could also affect CP RNA-binding activity, we deleted the codons of these residues in the pEHISTEV-TVBMVCP vector to produce pEHISTEV-TVBMVCP^{S129del}, pEHISTEV-TVBMVCP^{P130del}, pEHISTEV-TVBMVCP^{R161del}, pEHISTEV-TVBMVCP^{Y188del}, pEHISTEV-TVBMVCP^{D205del}, pEHISTEV-TVBMVCP^{A228del}, and pEHISTEV-TVBMVCP^{V229del}. His-TVBMVCP, His-TVBMVCP^{P130del}, His-TVBMVCP^{A228del}, and His-TVBMVCP^{V229del} were successfully expressed in *E. coli* cells, whereas His-TVBMVCP^{S129del}, His-TVBMVCP^{R161del}, His-TVBMVCP^{Y188del}, and His-TVBMVCP^{D205del} failed and were excluded in subsequent analyses (Supplementary Fig. S4). The EMSA results showed that the binding signal between the RNA probe and His-TVBMVCP was gradually increased as the concentration of CP was increased from 0 to 2.4 μM but not the binding signal between the RNA probe and His-TVBMVCP^{P130del}, His-TVBMVCP^{A228del}, or His-TVBMVCP^{V229del} (Fig. 7C). This result suggested that these residues were also crucial for TVBMV cell-to-cell movement and CP RNA-binding activity.

The corresponding nine residues in the WMV CP RNA-binding pocket were also critical for virus cell-to-cell movement.

To investigate whether the corresponding residues in the WMV CP RNA-binding pocket were also involved in virus cell-to-cell movement, we analyzed the 3D structure of the WMV CP-RNA complex (Zamora et al. 2017) and found that nine residues (i.e., S¹⁴⁰, P¹⁴¹, R¹⁷², Y¹⁹⁹, R²⁰³, D²¹⁶, K²³⁶, A²³⁹, and L²⁴⁰) were within 4 Å distance to the nucleotide U4 in the RNA-binding pocket (Fig. 8A). These residues corresponded to the nine residues (S¹²⁹, P¹³⁰, R¹⁶¹, Y¹⁸⁸, R¹⁹², D²⁰⁵, K²²⁵, A²²⁸, and V²²⁹) in the TVBMV CP RNA-binding pocket. We then made nine deletion mutants using pCBWMV-GFP (WMV-GFP) as the backbone to produce plasmids pCBWMV^{S140del}-GFP,

Fig. 4. Effects of R¹⁹² and K²²⁵ mutations on coat protein (CP) RNA-binding activity determined by electrophoretic mobility shift assay. Increased amounts (0, 0.6, 1.2, and 2.4 μM) of His-tobacco vein banding mosaic potyvirus (TVBMV) CP or its mutants were incubated with 8 pmol hexafluorescein (HEX)-labeled RNA probe in each reaction. The RNA probe sequence was selected from the CP open reading frame sequence. Reactions were separated in native polyacrylamide gels through electrophoresis. The fluorescence signal of the HEX-labeled RNA probe was captured using a multicolor fluorescence imaging system. Positions of the CP-RNA probe complexes and free RNA probe were indicated by arrowheads (upper panel). Increased amounts of His-TVBMVCP, His-TVBMVCP^{R192A}, His-TVBMVCP^{K225A}, and His-glutathione S-transferase (GST) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Gels were stained with Coomassie brilliant blue R-250 (bottom panel).

pCBWMV^{P141del}-GFP, pCBWMV^{R172del}-GFP, pCBWMV^{Y199del}-GFP, pCBWMV^{R203del}-GFP, pCBWMV^{D216del}-GFP, pCBWMV^{K236del}-GFP, pCBWMV^{A239del}-GFP, and pCBWMV^{L240del}-GFP, respectively. Agrobacterial cultures (OD₆₀₀ = 0.0001) harboring individual plasmids were infiltrated into *N. benthamiana* leaves. At 5 dpai, GFP fluorescence was

observed in many cells per locus in the WMV-GFP-inoculated *N. benthamiana* leaves, while the GFP fluorescence was observed in single cells in the *N. benthamiana* leaves inoculated with individual deletion mutants (Fig. 8B). These results showed that these nine residues also played essential roles in WMV cell-to-cell movement.

Fig. 5. Effects of R¹⁹² and K²²⁵ mutations on coat protein (CP) plasmodesmata localization. Colocalization of **A**, green fluorescent protein (GFP)-tobacco vein banding mosaic potyvirus (TVBMV) CP; **B**, GFP-TVBMVCP^{R192A}; or **C**, GFP-TVBMVCP^{K225A} with CI-DsRed in the periphery of *Nicotiana benthamiana* leaf cells, in the presence of TVBMV or its mutants' infection and N terminus of P3 and residues encoded by pretty interesting potyvirus open reading frame (P3N-PIPO). White arrows indicated the punctate of GFP-TVBMVCP, GFP-TVBMVCP^{R192A}, or GFP-TVBMVCP^{K225A} and CI-DsRed. Images were taken at 48 h postagroinfiltration under a confocal microscope. Bars = 5 μ m.

Fig. 6. Transcomplementation of cell-to-cell movement-defective tobacco vein banding mosaic potyvirus (TVBMV) mutants. **A**, Schematic diagram of the plasmids pCBTVBMV^{R192A}-GFP//mCherry, pCBTVBMV^{K225A}-GFP//mCherry, pCamMyc-TVBMVCP^{WT}, pCamMyc-TVBMVCP^{R192A}, and pCamMyc-TVBMVCP^{K225A}. UTR = untranslated region. **B**, Green fluorescent protein (GFP) and mCherry fluorescence in *Nicotiana benthamiana* cells inoculated with different combinations of agrobacteria harboring proper plasmids as indicated. The agrobacterial culture harboring pCBTVBMV^{R192A}-GFP//mCherry or pCamTVBMV^{K225A}-GFP//mCherry was adjusted to an optical density at 600 nm (OD_{600}) = 0.0003, whereas cultures harboring other plasmids were adjusted to OD_{600} = 0.6. All agrobacterial cultures were mixed at equal ratios. Infiltrated leaf tissues were examined at 5 days postagroinfiltration (dpi). Bars = 50 μ m. **C**, Numbers of cells showing GFP fluorescence in each infection locus per treatment were obtained at 5 dpi. The value of each column represents the means from 30 infection loci and bars indicate the positive and negative standard deviations. Statistical differences were determined by the Duncan's multiple range test ($P < 0.05$). The same lowercase letters indicated no significant differences between the treatments. **D**, Confirmation of expression of Myc-TVBMVCP^{WT}, Myc-TVBMVCP^{R192A}, and Myc-TVBMVCP^{K225A} via Western blotting assay. Ponceau red-stained Rubisco large subunit (Rubi) was used to show sample loadings. Ab = antibody.

DISCUSSION

In this study, we have determined the critical residues that are located in the RNA-binding pocket of TVBMV and WMV CP, and found that these residues are involved in the viral cell-to-cell movement, replication, and the RNA-binding activity.

RNA-binding sites have been identified in various virus CPs. Using a reversible formaldehyde cross-linking method and mass spectrometry, Hung et al. (2014) found that two arginine residues (R⁹⁹ and R²²⁷) in the CP of bamboo mosaic virus (BaMV, genus *Potexvirus*) are involved in RNA binding. In a separate study, the phosphorylation sites in the T²⁴²TSEED²⁴⁷ motif in the PVA CP core region were found to be critical for the CP RNA-binding activity (Ivanov et al. 2003). Analysis of the 3D model of pepino mosaic virus (PepMV, genus *Potexvirus*) CP showed that residues K⁹³, R¹²⁴, D¹⁶³, and K¹⁹⁶ could form RNA-binding sites (Agirrezabala et al. 2015). Analyses of the 3D models of WMV, PVY, and TuMV CPs have also shown that residues S¹²⁹, R¹⁶¹, and D²⁰⁵ could form RNA-binding sites (Cuesta et al. 2019; Kežar et al. 2019; Zamora et al. 2017). It was reported that the presence of at least one atom in the protein with a distance cutoff <5 Å from an atom of the binding RNA can be considered to be an RNA-binding site (Terribilini et al. 2007; Wang and Brown 2006; Tang et al. 2017). In this study, we found nine residues (i.e., S¹²⁹, P¹³⁰, R¹⁶¹, Y¹⁸⁸, R¹⁹², D²⁰⁵, K²²⁵, A²²⁸, and V²²⁹) in the TVBMV CP with less than 4 Å from the nucleotide U4, and these residues are all located in the RNA-binding pocket (Figs. 1 and 8). Sequence alignment showed that these residues, except V²²⁹, were conserved in potyvirus CPs (Supplementary Fig. S5), suggesting that the CP RNA-binding sites in potyvirus CP are conserved.

During virus infection in the plant, virus RNA is transported from the originally infected cells into the neighboring cells with the assistance of viral proteins with RNA-binding activities. Therefore, mutations of residues R⁹⁹ and R²²⁷ in the BaMV CP

RNA-binding site inhibited virus cell-to-cell movement (Hung et al. 2014). Residues K⁹³, R¹²⁴, D¹⁶³, and K¹⁹⁶ in the PepMV CP RNA-binding site were also considered to be crucial for virus cell-to-cell movement (Agirrezabala et al. 2015). However, no experimental evidence is currently available to link the potyvirus CP RNA-binding activity with virus cell-to-cell movement. Here, we have shown that substitution of the positively charged residues R¹⁹² or K²²⁵ in TVBMV CP to residues with different properties reduced the CP RNA-binding activity and abolished virus cell-to-cell movement in *N. benthamiana* plant (Figs. 2, 3, and 4). Because TVBMV RNA was not present in the purified VLPs of TVBMV^{R192A}-GFP and TVBMV^{K225A}-GFP (Supplementary Fig. S3), it is possible that these RNA-free VLPs were formed due to the reduced CP RNA-binding activity. We have also found that deletions of the other seven residues in the TVBMV CP RNA-binding pocket reduce CP RNA-binding activity and abolish TVBMV cell-to-cell movement, and deletions of the corresponding residues in the WMV CP also abolished virus cell-to-cell movement (Figs. 7 and 8). These results indicate that the nine residues within the CP RNA-binding pocket are involved in the potyvirus cell-to-cell movement through binding to viral RNA. It is also possible that deletions of these residues individually may change the 3D structure of CP or the RNA-binding pocket, leading to the reduction of CP RNA-binding activity.

The replication and cell-to-cell movement of potyviruses are two coupled processes (Chai et al. 2020; Cui and Wang 2016; Deng et al. 2015). The phosphorylation site T²⁴³ in the PVA CP core region is known to be involved in the shifting from translation to replication through regulation of RNA-binding activity (Ivanov et al. 2003; Löhmus et al. 2017). The TEV CP coding sequence encompassing the codons for residues 138 to 189 and residues 211 to 246 is known to be involved in TEV replication (Haldeman-Cahill et al. 1998; Mahajan et al. 1996). PVY

Fig. 7. Effects of deletions of the other seven residues (S¹²⁹, P¹³⁰, R¹⁶¹, Y¹⁸⁸, R¹⁹², D²⁰⁵, K²²⁵, A²²⁸, and V²²⁹) within the coat protein (CP) RNA-binding pocket of CP on tobacco vein banding mosaic potyvirus (TVBMV) cell-to-cell movement and the RNA-binding activity of CP. **A**, Cell-to-cell movement of TVBMV-green fluorescent protein (GFP) and its mutants with S¹²⁹, P¹³⁰, R¹⁶¹, Y¹⁸⁸, R¹⁹², D²⁰⁵, K²²⁵, A²²⁸, or V²²⁹ deletions. GFP fluorescence in the *Nicotiana benthamiana* leaf cells infected with TVBMV-GFP or one of its mutants was captured under a confocal microscopy at 5 days postagroinfiltration (dpi). **B**, Numbers of cells showing GFP fluorescence per infection locus in the *N. benthamiana* leaves infiltrated with different mutants at 5 dpi. The value of each column represents the means from 30 infection loci and bars indicate the positive and negative standard deviations. Statistical differences were determined by the Duncan's multiple range test ($P < 0.05$). The same lowercase letters indicated no significant differences between the treatments. **C**, RNA-binding activity of His-TVBMVCP and its mutants with P¹³⁰, A²²⁸, or V²²⁹ mutation. Electrophoretic mobility shift assay was performed with 8 pmol hexafluorescein-labeled RNA probe and an increased amount (0, 0.6, 1.2, and 2.4 μM) of His-TVBMVCP or one of its mutants. The detection signal was captured using a multicolor fluorescence imaging system. Arrowheads indicate the positions of the free RNA probe and the CP-RNA probe complexes (upper panel). Gels stained with Coomassie brilliant blue R-250 were used to show sample loadings (bottom panel).

mutants lacking the C-terminal 60 residues had extremely low replication levels (Kežar et al. 2019). However, deletion of almost the entire coding region for TuMV CP did not affect virus replication (Dai et al. 2020). Here, we have shown that the replications of TVBMV with mutations in the CP RNA-binding pocket were significantly reduced (Fig. 3). This defective replication is likely caused by the weakened CP RNA-binding activity or the mutated virus cell-to-cell movement, or the mutated RNA sequence. We are unable to exclude the possibility that the reduced replication was caused by the reduced virus cell-to-cell movement. Further experiments are still needed to elucidate how the interaction between CP and viral RNA affected viral replication and cell-to-cell movement.

Both the core domain and C domain of potyvirus CP have been shown to affect virus cell-to-cell movement in plants (Dai et al. 2020). CP is also necessary for virion formation and trafficking of the viral ribonucleoprotein complex into adjacent cells via PDs (Chai et al. 2020; Geng et al. 2015; Wei et al. 2010). In this study, because the CP with R¹⁹² or K²²⁵ mutations also localized at PDs and formed RNA-free VLPs (Fig. 5; Supplementary Fig. S3), we hypothesized that these mutations might affect the ability of CP to bind and package viral RNA to produce infectious ribonucleoprotein complex or virion, which leads to the reduced TVBMV replication and cell-to-cell movement.

To sum up, we have shown that residues R¹⁹² and K²²⁵ in the CP RNA-binding pocket are critical in viral RNA binding, which influences both TVBMV cell-to-cell movement and replication. Our results provide experimental evidence to link the CP RNA-binding activity with potyvirus cell-to-cell movement and replication. Further studies are still needed to elucidate how RNA-binding activity orchestrates potyvirus replication and cell-to-cell movement.

MATERIALS AND METHODS

TVBMV CP 3D modeling.

A 3D model of TVBMV CP was predicted using the I-TASSER software with default parameters (Yang et al. 2015), and then the PyMOL Molecular Graphics System.

Plasmid construction and mutagenesis.

Infectious clones pCamTVBMV (accession number JQ407082) without a *GFP* gene, pCamTVBMV-GFP with a *GFP* gene, and

pCBWMV-GFP (accession number MN910314) were constructed in our laboratory (Gao et al. 2012; Geng et al. 2015) (unpublished data). The 35S-TVBMV-GFP-Nos cassette was PCR amplified from pCamTVBMV-GFP and inserted into the expression vector pCBmCherry to produce pCBTVBMV-GFP//mCherry. Coding sequences of CP and P3N-PIPO were PCR amplified from pCamTVBMV and inserted into the expression vector pCam35S to produce pCamP3N-PIPO and pCamMyc-TVBMVCP^{WT} for expressing nontagged P3N-PIPO and Myc-tagged TVBMV CP, respectively. The TVBMV CP coding sequence was also cloned into pCam35S::GFP to produce pCamGFP-TVBMVCP for expressing GFP-TVBMV CP fusion. The TVBMV CI coding sequence was PCR amplified and cloned into pCam35S::DsRed to produce pCamCI-DsRed for expressing CI-DsRed fusion. The TVBMV CP coding sequence was cloned into pEHISTEV to produce pEHISTEV-TVBMVCP for expressing His-TVBMVCP in *E. coli* Rosetta cells. The plasmid pBinP19 was used to express P19 of tomato bushy stunt virus (Himber et al. 2003). Mutants used in this study were made via a site-directed mutagenesis approach (Liu and Naismith 2008) using the primers listed in Supplementary Table S1. All of the plasmids were sequenced before use.

Plant growth, virus inoculation, and protein transient expression.

N. benthamiana plants were grown in a greenhouse at 25°C under a photoperiod of 16 h of light and 8 h of darkness. Agrobacterial cultures harboring specific plasmids were infiltrated into fully expanded leaves of *N. benthamiana* plants with 1-ml needleless syringes. The agrobacterial cultures were infiltrated at varied concentrations, as indicated in the content.

RNA extraction and qRT-PCR.

Assayed leaf tissues of *N. benthamiana* plants were collected for total RNA extraction using TransZol reagent (TransGen Biotech, Beijing, China) following the manufacturer's instructions. qRT-PCR was performed as described previously (Yan et al. 2021). After gDNA wiper Mix (Vazyme, Nanjing, China) treatment, total RNA (500 ng per sample) was reverse transcribed using a HiScript II Q RT SuperMix kit (Vazyme) with random or specific primers. Quantitative PCR was then performed with a ChamQ SYBR qPCR Master Mix (Vazyme) on

Fig. 8. Effects of mutations of residues in the watermelon mosaic virus (WMV) coat protein (CP) RNA-binding pocket on virus cell-to-cell movement. **A**, Positions of the nine residues (S¹⁴⁰, P¹⁴¹, R¹⁷², Y¹⁹⁹, R²⁰³, D²¹⁶, K²³⁶, A²³⁹, and L²⁴⁰) in the WMV CP RNA-binding pocket. The three-dimensional model of WMV CP is presented as a white-ribbon model (accession code 5ODV). The polyU RNA is indicated as green, and the nucleotide U4 of the polyU is shown as magenta. Residues S¹⁴⁰, P¹⁴¹, R¹⁷², Y¹⁹⁹, R²⁰³, D²¹⁶, K²³⁶, A²³⁹, and L²⁴⁰ are located within 4 Å of the nucleotide U4 and are shown in blue, cyan, orange, tint, and yellow. **B**, Numbers of cells with green fluorescent protein (GFP) fluorescence per infection locus produced by WMV-GFP mutants. The GFP fluorescence in *Nicotiana benthamiana* leaf cells infected with WMV-GFP and one of its mutants was observed under a confocal microscope at 5 days postagroinfiltration. The value of each column represents the means from 30 infection loci and bars indicate the positive and negative standard deviations. Statistical differences were determined by the Duncan's multiple range test ($P < 0.05$). The same lowercase letters indicated no significant differences between the treatments.

a real-time PCR system (LC96; Roche, Basel, Switzerland). The expression of the *N. benthamiana* *EF1α* gene (Niben101Scf04639g06007.1) served as an internal control. Primers used in this study are listed in Supplementary Table S1.

ELISA.

GFP- or TVBMV CP-specific antibody was used as the primary antibody. An alkaline phosphatase-conjugated goat antirabbit immunoglobulin G (IgG) (Sigma-Aldrich, St. Louis, MO, U.S.A.) was used as the secondary antibody. p-Nitrophenyl phosphate was used as the substrate as instructed (Sigma-Aldrich). The optical density of each sample at 405 nm was measured using a Multifunction Microplate Reader (BioTek Synerg Mx, Winooski, VT, U.S.A.).

Protein expression and purification.

E. coli Rosetta cells transformed with pEHISTEV-TVBMVCP or various mutant constructs were individually cultured till $OD_{600} = 0.6$. The cultures were added with 0.1 mM isopropyl- β -D-thiogalactopyranoside, then incubated at 22°C for 16 h. The cells were harvested by centrifuging at $8,000 \times g$ for 2 min. The pellets were resuspended in a lysis buffer (50 mM Na_2HPO_4 , 300 M NaCl, and 10 mM imidazole, pH 8.0), incubated for 15 min on ice, and lysed through 15 min of sonication on ice. The lysed cells were pelleted through 20 min of centrifugation at $10,000 \times g$. The supernatant of each sample was transferred into a new tube, mixed with 500 μ l of nickel-nitrilotriacetic acid resin (Genscript, Wuhan, China), and incubated at 4°C for 1 h. The resulting mixtures were loaded individually onto chromatographic columns (Beyotime, Beijing, China) and each column was washed with 5 ml of washing buffer (50 mM Na_2HPO_4 , 300 M NaCl, and 30 mM imidazole, pH 8.0). Proteins maintained in columns were eluted using an elution buffer (50 mM Na_2HPO_4 , 300 M NaCl, and 250 mM imidazole, pH 8.0).

EMSA.

Because potyvirus CP binds RNA in a sequence-unspecific manner, we used a fragment of TVBMV CP coding sequence (5'-⁷²¹GGA AAU GUC AGC ACG AAG GAG GAA AAC ACA⁷⁵⁰-3') as an RNA probe. This synthetic RNA probe was labeled with HEX (Sangon Biotech, Shanghai, China). Each purified CP sample was incubated with the HEX-labeled RNA probe in a 20- μ l binding buffer (150 mM KCl, 0.1 M dithiothreitol, 0.02 mM EDTA, and 10 mM Tris, pH 7.4) for 20 min at room temperature. The reaction mixtures were then separated in 12% native polyacrylamide gels through electrophoresis. The fluorescence signal from the HEX-labeled RNA probe was captured using a multicolor fluorescence imaging system (Sage, Beijing, China).

Confocal microscopy.

The assayed leaves were harvested at various hours or days postagroinfiltration and examined under a confocal microscope (Carl Zeiss, Oberkochen, Germany). The excitation and emission wavelengths for GFP fluorescence observations were set at 488 and 520 to 540 nm, respectively, and those for DsRed were set at 561 and 590 to 630 nm, respectively. The captured images were further processed using the ZEN 2.1 software (Carl Zeiss).

Virus inoculation, virus particle purification, and viral RNA extraction.

N. benthamiana leaves were inoculated with wild type or mutant TVBMV through agroinfiltration. Various virus-inoculated leaf samples were harvested, ground in liquid nitrogen, homogenized in 0.2 M phosphate buffer, pH 8, containing 0.15% β -mercaptoethanol and 0.01 M EDTA, and incubated for 20 min

at 4°C. Each sample was centrifuged again at $8,000 \times g$ for 20 min. After the addition of 1% Triton X-100, polyethylene glycol 6000 at 40 g/liter and 0.2 M NaCl to the supernatant, the samples were stirred for 3 h at 4°C. The sample was centrifuged again at $8,000 \times g$ for 20 min and the pellet was resuspended overnight at 4°C in 0.2 M phosphate buffer, pH 8.0, containing 1% Triton-X 100. The sample was centrifuged at $8,000 \times g$ for 20 min and the supernatant was centrifuged at $100,000 \times g$ for 1 h at 4°C in a CP100WX ultracentrifuge (Hitachi, Tokyo, Japan). The pellet was resuspended overnight in 0.05 M phosphate buffer, pH 8, at 4°C. After insoluble materials were removed from each sample through low-speed centrifugation, a small aliquot from each sample was loaded onto 230-mesh carbo-coated copper grids, stained with 2% uranyl acetate, and examined under a JEM-1200Ex transmission electron microscope for virus particles (Jeol, Tokyo).

The purified virus particle samples were also treated with the gDNA wiper Mix (Vazyme) at 42°C for 2 min and then RNase A (100 μ g/ml) (Omega Bio-Tek, Norcross, GA, U.S.A.) at 37°C for 10 min. Removal of DNA and RNA from these samples was confirmed through PCR and RT-PCR. The treated virus particle samples were extracted using Trizol reagent (TransGen Biotech) for viral RNA followed by RT-PCR detection. The RT reaction was performed using a HiScript II Q RT SuperMix kit (Vazyme) and random primers. The PCR was performed using a *Taq* DNA polymerase (Vazyme) with primers TVBMV-CP-F and TVBMV-CP-R.

Western blotting assay.

Total protein was extracted from *N. benthamiana* leaf tissues using an extraction buffer (100 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10% glycerol, 0.1% NP40, and 1 mM phenylmethanesulfonyl fluoride) at a ratio of 1:2. The extracted proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and were then transferred to nitrocellulose membranes. The 1:2,000 (vol/vol) diluted Myc antibody (Abways, Shanghai, China) was used as the primary antibody and the 1:50,000 (vol/vol) diluted horseradish peroxidase-conjugated goat antirabbit IgG (Sigma-Aldrich) was used as the secondary antibody.

Amino acid sequence alignments.

The CP sequences were aligned using the online software WebLogo to produce a sequence logos as described (Crooks et al. 2004).

AUTHOR-RECOMMENDED INTERNET RESOURCE

N. benthamiana *EF1α* gene:
https://solgenomics.net/organism/Nicotiana_benthamiana/genome

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