1   **Modulation of Capsid Dynamics in Bromoviruses by the Host and**

2   **Heterologous Viral Replicase**

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10   **Running title**: Modulation of capsid dynamics in bromoviruses

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ABSTRACT

The tripartite genome of *Cowpea chlorotic mottle virus* is packaged into three morphologically indistinguishable icosahedral virions with T=3 symmetry. The two virion types, $C1^V$ and $C2^V$, package genomic RNAs 1 (C1) and 2 (C2), respectively. The third virion type, $C3+4^V$, co-packages genomic RNA3 and its sub-genomic RNA (RNA4). In this study, each virion type was separately assembled *in planta* using a previously established agroinfiltration approach. The three virion types are indistinguishable under the EM and by electrophoretic mobility. However, the stability and the capsid dynamics evaluated respectively by the differential scanning fluorimetry and MALDI-TOF analysis following trypsin digestion distinguished the three virions type. Proteolytic analysis revealed that, while retaining the structural integrity, $C1^V$ and $C2^V$ virions released peptide regions encompassing the N-terminal arginine-rich RNA binding motif. In contrast, a minor population of the $C3+4^V$ virion type was sensitive to trypsin-releasing peptides encompassing the entire capsid protein region. In addition, we also evaluated the effect of the two host organisms on the capsid dynamics. The wild-type CCMV virions purified from cowpea are highly susceptible to trypsin digestion, while those from *N. benthamiana* remained resistant. Finally, the MALDI-TOF evaluated the relative dynamics of $C3+4^V$ and $B3+4^V$ virions assembled under the control of the homologous vs. heterologous replicase. The significance of the viral replicase in modulating the capsid dynamics was evident by the differential sensitivity to protease exhibited by $B3+4^V$ and $C3+4^V$ virions assembled under the homologous vs. heterologous replicase. The significance of these results in relation to viral biology is discussed.

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47 **IMPORTANCE**

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49 Infectious virus particles or virions are considered static structures and undergo various

50 conformational transitions to replicate and infect many eukaryotic cells. Although viral

51 capsid fluctuations, referred to as dynamics or breathing, have been well studied in

52 RNA viruses pathogenic to animals, such information is limited among plant viruses.

53 *Cowpea chlorotic mottle virus* (CCMV) and Brome mosaic virus (BMV), RNA viruses

54 pathogenic to plants, contain a segmented genome distributed among three physically

55 and structurally homogenous icosahedral virion types. This study examines the role of

56 the host organism and the heterologous viral replicase in modulating the thermal

57 stability and capsid dynamics in CCMV and BMV. The results accentuate that alteration

58 of the capsid dynamics by the host type and heterologous replicase are likely to affect

59 the biology of the virus.

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61 **Key words:** Capsid dynamics, Stability, genome packaging, RNA virus, MALDI-TOF

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INTRODUCTION

71   The assembly of viral capsids is promoted and directed by the interaction

72   between the capsid protein (CP) subunits and the viral nucleic acid in both sequence-

73   specific and non-specific manner with the assistance of other scaffolding proteins (1, 2).

74   Once matured, the primary function of a viral capsid is to encapsidate the viral genome

75   and remain stable enough to withstand the physicochemical environmental conditions

76   until a susceptible host cell is encountered. In addition, assembled viral capsids must be

77   flexible enough to fulfill multifunctional roles such as receptor binding, cellular entry,

78   navigating the intracellular environment, and release of the viral genome. To meet these

79   requirements, viral capsids are conformationally dynamic assemblies fluctuating from

80   inside to outside of the particle (2). These fluctuations or the protein subunits' dynamism

81   manifest the breathing of the virus particles that position the biologically relevant surface

82   peptides critical for their infectivity (3).

83   The three single-stranded, positive-sense genomic RNA molecules of the two

84   closely related members of the family *Bromoviridae,* namely *Cowpea chlorotic mottle*

85   *virus* (CCMV; C1, C2, and C3) and *Brome mosaic virus* (BMV; B1, B2, and B3) are

86   encapsidated separately into three individual virions (4). The two largest monocistronic

87   genomic RNAs of CCMV and BMV- RNA1 (C1, 3171 nt and B1, 3237 nt) and- RNA2

88   (C2, 2774 nt and B2, 2864 nt), encoding nonstructural replicase proteins 1a

89   (methyltransferase/helicase-like)   and   2a   (RNA-dependent   RNA   polymerase)

90   respectively, are packaged separately into two virion types, $C1^V/B1^V$ and $C2^V/B2^V$ (4).

91   The dicistronic genomic RNA3 (C3 and B3) encodes a nonstructural movement protein

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92   (MP) at its 5' and a structural CP at its 3' end. In these two viruses, CP is translated

93   from a replication-derived subgenomic RNA4 (C4 and B4) via an internal initiation

94   mechanism from the genomic progeny minus-strand RNA3. Genomic RNA3 and

95   subgenomic RNA4 (sgRNA4) are co-packaged into a third virion type: C3+4^V and B3+4

96   (5).

97   BMV and CCMV exhibit similar genome organization and replication mechanisms

98   (6, 7). However, they do differ in many properties, including the required virus-encoded

99   genes for cell-to-cell spread (8), electrophoretic mobility patterns of respective virions

100 (9), and finally, the interaction of highly conserved N-terminal basic arm region of the

101 CP with respective genomic and subgenomic RNAs during encapsidation (10, 11). The

102 CPs of CCMV and BMV are 190 and 189 amino acids in length (aa), respectively, and

103 are 70% homologous. In these two viruses, the first N-terminal 25 aa region of the CP is

104 not visible in the crystal structure since this highly basic region projects into the interior

105 region interacting with the RNA, while the remainder of the CP is highly structured (4).

106 The interaction between amino and carboxyl termini is essential for forming CP dimers,

107 the building blocks for the virus assembly (12).

108   Fully assembled virions of CCMV and BMV exhibit icosahedral morphology with

109 a diameter of ~28 nm and T=3 quasi-symmetry (13, 14). Although the three genomic

110 and a single subgenomic RNA are distributed disproportionately among three virions,

111 they sediment as a single peek and are physically indistinguishable by any known

112 procedures. Recently, we applied an *Agrobacterium*-mediated transient expression

113 system (agroinfiltration) to independently assemble each of the three virion types of

114   BMV (9). Application of limited proteolysis in conjunction with MALDI-TOF analysis

115   revealed that B1^V and B2^V virions are distinct from the B3+4^V in their stability and

116   dynamics, suggesting that RNA-dependent capsid dynamics play an important

117   biological role in the viral life cycle.

118   Although at biochemical and structural levels, CCMV and BMV are similar, at

119   biological and genetic levels, they exhibit the following two differential traits (i)

120   Inherently, CCMV and BMV are respectively adapted to dicotyledonous Cowpea (*Vigna*

121   *sinensis*) monocotyledonous plants (brome grass); (ii) hybrids viruses involving the

122   exchange of the genomic RNA3, but not those encoding the replicase genes, are

123   biologically viable (15). Keeping these two differential traits in perspective, in this study,

124   we comparatively evaluated the following characteristics: (i) thermal stability and capsid

125   dynamics of each virion type of CCMV and compared to those of BMV; (ii) the influence

126   of the host organism on the dynamics of WT CCMV virions and finally (iii) thermal

127   stability and capsid dynamics of virions of B3+4^V and C3+4^V assembled under the

128   control of heterologous replicase. We observed a remarkable difference in the thermal

129   stability and capsid dynamics within and among the three virion types of CCMV and

130   BMV. In addition, we also observed that the type of host organism and heterologous

131   replicase have a profound influence on the dynamics of the assembled virions.

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133   **RESULTS**

134   Throughout this study, individual virions packaging the CCMV genomic

135   RNA1(C1), RNA2 (C2), and RNA3 (C3) plus subgenomic RNA4 (C4) are respectively

136   referred to as C1^V, C2^V, and C3+4^V. Likewise, those of BMV are designated as B1^V,

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137   B2^V, and B3+4^V. Wild type (Wt) represents a mixture of three virion types of either

138   CCMV or BMV, purified from infected plants.

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140   **Characteristic properties of the three individually assembled virion types of**

141   **CCMV (C1^V, C2^V, and C3+4^V).** Fig.1 (Panel IA) summarizes the characteristic features

142   of the transfer DNA (T-DNA)-based vectors engineered to express the three biologically

143   active genomic RNAs (gRNAs) of CCMV (pC1, pC2, and pC3). Fig. 1 (Panel IB)

144   summarizes the characteristic features of the plasmids engineered to transiently

145   express CCMV replicase proteins p1a (pC1a), p2a (pC2a), and the CP (pCCP). Using

146   these constructs, the strategy (Fig. 1, Panel II) used for assembling the three individual

147   virion types C1^V, C2^V, and C3+4^V, is essentially the same as that was described

148   previously (9) for assembling BMV virion types using the constructs described in Fig. 1

149   (Panel IC, D). The following five sets of the inocula resulted in the assembly of the three

150   virion types of CCMV *in planta* (i.e., C1^V, C2^V, and C3+4^V), _Set 1_: pC1+pC2+pC3 to

151   induce wild-type infection resulting in the accumulation of all three virion types that

152   served as a positive control (Fig. 1, Panel IIA); _Set 2_: pCCP to transiently express

153   CCMV CP only that served as a negative control (Fig. 1, Panel IIB); _Set 3_:

154   pC1+pC2a+pCCP to assemble virion type C1^V (Fig. 1, Panel IIC); _Set_

155   _4:_ pC1a+pC2+pCCP to assemble virion type C2^V (Fig. 1, Panel IID) and _Set 5_:

156   pC1a+pC2a+pC3 to assemble virion type C3+4^V (Fig. 1, Panel IIE).

157   Fig. 2 summarizes the progeny analysis from the five sets of inocula as

158   mentioned above. Western blot analysis (Fig. 2A) confirmed the efficient expression of

159   the monomeric and dimeric forms of the CCMV CP with expected size in plants

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160   infiltrated with each set (Fig. 2A, lanes 1-5). Next, virions purified from infiltrated leaves

161   were analyzed for their morphology and size by the EM and their relative electrophoretic

162   mobility pattern by agarose gel-electrophoresis (Fig. 2B, C). EM analysis (Fig. 2B)

163   revealed that virion preparations of C1^V, C2^{V,} and C3+4^V are indistinguishable from

164   those of Wt CCMV isolated from cowpea or *N. benthamiana* in morphology and size

165   (~28-30 nm). Finally, when electrophoresed in agarose gels, samples of C1^V, C2^V, and

166   C3+4^V virion types migrated as a single band (Fig. 2C). They were identical to that of Wt

167   CCMV (migrating toward positive polarity) and distinct from that of control virions of

168   BMV (migrating toward negative polarity). In addition, we also confirmed the nature of

169   the packaged RNA in C1^V, C2^V, and C3+4^V by RT-PCR (data not shown). Furthermore,

170   as expected, an inoculum containing all three virion types (i.e., C1^V+ C2^V + C3+4^V)

171   remained infectious to plants. None of the pair-wise combinations (i.e., C1^V+ C2^V or

172   C1^V+ C3+4^V or C2^V+ C3+4^V) remained non-infectious (data not shown). These results

173   confirmed the purity and the authenticity of the three virion types of CCMV assembled

174   independently in *N. benthamiana* plants.

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176   **Stability differences among C1^V, C2^V, and C3+4^V virions.** We previously observed

177   that differential scanning fluorimetry (DSF) is ideal for comparing the relative stability of

178   the three virion types in BMV (9). Fig. 3 (A-D) summarizes the results of the DSF

179   analysis of the temperature-dependent melting for C1^V, C2^V, and C3+4^V and control

180   samples of lysozyme under two buffer and pH conditions. First, we compared the

181   temperature-dependent melting of C1^V, C2^V, and C3+4^V. Although each virion type

182   revealed near identical thermal melting profiles displaying a single unstable peak at

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183   ~70°C in the virus suspension buffer (pH 4.5) (Fig. 3A), a clear distinction in the thermal

184   instability among the three virion types was evident in the phosphate buffer (pH 7.2).

185   For example, $C1^V$ and $C2^V$ displayed two major thermally stable population peaks (Fig.

186   3B; 46°C and 88°C for $C1^V$ and 50°C and 88°C for $C2^V$). By contrast, for $C3+4^V$, 80%

187   virion population showed instability at 48°C while the remaining 20% melted at 82°C

188   (Fig. 3B, compare two peaks of $C1^V$ and $C2^V$ vs. C3+4V). As expected, the melting

189   profile of the lysozyme control (Fig. 3 C, D) remained indistinguishable either in the virus

190   suspension buffer (pH 4.5) or phosphate buffer (pH 7.2). Interestingly, these melting

191   profiles of $C1^V$, $C2^V$, and $C3+4^V$ are distinct from those of BMV (see Discussion).

192   **Capsid dynamics of the three virion types of CCMV.** As observed in BMV (9), in

193   CCMV also, the three virion types ($C1^V$, $C2^V$, and $C3+4^V$) are accumulated

194   disproportionately (46% $C1^V$+$C2^V$ vs. 54% $C3+4^V$; data not shown). To verify whether

195   physically indistinguishable three virion types of CCMV are dynamically distinct, we

196   evaluated the capsid dynamics of each virion type by MALDI-TOF analysis following

197   trypsin digestion (16). Virion preparations of $C1^V$, $C2^V$, $C3+4^V$, and CCPV assembled

198   in *N. benthamiana* were concurrently subjected to trypsin digestion for 10, 30, 45 min,

199   and 3 hr. Then, each sample was divided into three equal aliquots and used for Western

200   blot, EM and MALDI-TOF analyses. Western blot identified the cleavage peptides, EM

201   analysis verified the structural integrity and MALDI-TOF identified the cleavage sites.

202   Undigested virion samples served as controls.

203   Fig. 4 (A, B) summarizes these results. Western blot data shown in Fig. 4A

204   revealed that undigested samples of the four individual virion types (i.e., $C1^V$, $C2^V$,

205    $C3+4^{V,}$ and CCPV) migrated as a single intact band with the expected molecular weight

206 (~20 kDa). Analysis of the trypsin cleavage products for each virus sample revealed

207 exciting profiles. Trypsin digestion of the three of four virion types, $C1^V$ and $C2^V$, and

208 CCP^V for 10, 30, and 45 min are indistinguishable from the undigested control samples

209 (Fig. 4A; Lanes 5, 6, 8 for 10 min; lanes 9, 10, 12 for 30 min and lanes 13, 14, and 16

210 for 45 min). By contrast, for the $C3+4^V$ virion type, although a majority (>50%) of the

211 protein remained intact, several faster-migrating peptide fragments were consistently

212 detected (Fig. 4A, lanes 7, 11, and 15). Extending the trypsin digestion time to 3h did

213 not change these profiles (Table 1). EM analysis of the trypsin-digested virion

214 preparations (Fig. 4B) confirmed the Western blot data, showing that most of the $C1^V$

215 and $C2^V$ (Fig, 4B), as well as CCP (data not shown), remained structurally intact.

216 Whereas for $C3+4^V$ virions, the majority of the virions remained intact, while structurally

217 disrupted virions are also visible (Fig, 4B).

218   Fig. 5 (A, B) summarizes the organization of the three CP subunits (A, B, and C)

219 on the surface of the CCMV virion and T=3 lattice. Fig. 5 (C-E) summarizes the position

220 of the trypsin cleavage sites located on each CP subunit.   The deconvoluted mass

221 spectrum obtained from the total ion chromatogram (TIC) of $C1^V$, $C2^V$, and $C3+4^V$

222 indicated that all the virions are assembled from a single protein of 20 kDa (data not

223 shown). Analysis of the peptides released by the trypsin digestion at early (10 min) and

224 late time points (45 min and 3 hr) distinguished virions of $C1^V$ and $C2^V$ from those of

225 $C3+4^V$ (Fig 5 F-H).   For example, 10 min after the digestion, $C1^V$ and $C2^V$ virions

226 appeared to release a small proportion of the peptides corresponding to aa

227 encompassing the N-ARM region (e.g., aa 2-15; Fig. 5F; Table 1). Since the N-proximal

228 region is predicted to be mobile/disordered and internalized following its interaction with

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229   RNA (17) and are not ordered in the crystal structure, their cleavage did not affect the

230   structural integrity of most of the $C1^V$ and $C2^V$ virions, as seen under EM (Fig. 4B).

231   Also, there were peaks of very low-intensity corresponding to aa 83-87 and 85-90

232   (Table 1). These fragments could have been generated from a very small percentage of

233   virions, hence not detected in Western blot. The trypsin digestion to 45 min and 3 hr of

234     $C1^V$ and $C2^V$ released several other aa fragments corresponding to additional cleavage

235   sites on the virion surface (Table 1), including K65 (Fig. 5 C-E). These fragments are

236   present at a low % intensity and hence are not detectable by the Western blot (Fig. 4A).

237   In contrast, irrespective of the time of incubation, virions of $C3+4^V$ remained

238   susceptible to trypsin digestion. As early as 10 min, virions of $C3+4^V$ released multiple

239   peptides encompassing the entire CP region (Fig. 5 H). This digestion pattern did not

240   change significantly at later time points (45 min and 3 hr). Mass mapping identification

241   of the released peptides revealed that the accessible trypsin cleavage sites (e.g., K45,

242   K54, K65, K87, K106, and K131; Fig. 5 C-E) (Table 1) were consistent with the reported

243   surface structure of the CCMV virions (http://viperdb.scripps.edu/). Given the

244   importance of the C-terminal peptide region in the virus assembly (16), these

245   observations explain why some of the virions of $C3+4^V$ appeared degraded under EM

246   (Fig. 4B). Taken together, it is reasonable to conclude that a percentage of trypsin-

247   digested $C3+4^V$ samples show qualitatively distinct capsid dynamics from those of $C1^V$

248   and $C2^V$, exhibiting a different conformational arrangement of the CP amino acid

249   residues in the virion exterior.

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251 **Effect of the host type on the stability of CCMV virions.** For CCMV, cowpea is the

252 natural host, while *N. benthamiana* is a symptomless experimental host (18). Since the

253 type of host plant can have a profound influence on virion phenotype (19), we wanted to

254 examine the effect of the host plant on the stability and dynamics of CCMV virions.

255 Consequently, we compared the stability of CCMV virions purified from cowpea and *N.*

256 *benthamiana* plants using DSF and dynamics by MALDI-TOF following trypsin

257 digestion. For the first assay, CCMV virions purified from cowpea and *N.*

258 *benthamiana* were suspended in either virus suspension buffer (pH 4.5) or phosphate

259 buffer (pH 7.2) and subjected to DSF analysis. Fig. 6A summarizes these results. In

260 virus suspension buffer (pH 4.5), most of the Wt CCMV virion population purified from

261 cowpea and *N. benthamiana* plants displayed a single melting peak at 65°C and 69°C,

262 respectively . In addition, virions purified from *N. benthamiana* but not from cowpea

263 plants (Fig. 6A, left panel) revealed a small melting population appearing as a shoulder

264 peak at ~50°C. By contrast, DSF analysis in the phosphate buffer (pH 7.2) revealed a

265 contrasting scenario (Fig. 6A, right panel). For example, unlike virus suspension buffer

266 (pH 4.5), CCMV virions from cowpea plants displayed a significant peak at 46°C and a

267 minor peak at 87°C (Fig. 6A, right panel). In contrast, CCMV virions obtained from *N.*

268 *benthamiana* plants displayed two significant peaks with equal intensity, one at 45°C

269 and the other at 79°C (Fig. 6A, right panel).

270 Next, we evaluated the relative susceptibility of Wt CCMV virions from cowpea

271 and *N. benthamiana* to trypsin digestion as described in the Methods section.

272 Undigested virion preparation served as control, and Fig. 6B summarizes these results.

273 When compared to the undigested control sample (Fig. 6B, lanes 1 and 5), Wt CCMV

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274   virions from cowpea plants are susceptible to trypsin digestion as early as 10 min (Fig.

275   6B, lanes 2-4), while those from *N. benthamiana* plants remained stable even after 45

276   min (Fig. 6B, lanes 6-8). EM analysis (Fig. 6C) confirmed these observations. To

277   identify the peptide fragments, MALDI-TOF analysis was performed for Wt CCMV virion

278   from cowpea and *N. benthamiana*. Results are shown in Fig. 6D. As expected, Wt

279   CCMV virions from cowpea released peptide fragments encompassing the entire CP

280   region (Fig. 6D, left panel). Although Western blot analysis failed to detect any cleavage

281   products when Wt CCMV virions from *N. benthamiana* were subjected to trypsin

282   treatment, MALDI-TOF revealed a profile similar to that of CCMV virions from cowpea

283   plants. However, these peptides appeared to be weaker in intensity and hence are not

284   detected by the Western blot analysis. No change in the MALDI-TOF profile was

285   observed with prolonged incubation with trypsin (data not shown). These results

286   suggest, CCMV virions in *N. benthamiana* accumulate as two virion populations: a

287   minor population susceptible to trypsin and a major trypsin resistant population. These

288   results collectively accentuate that the host plant type has a profound influence on the

289   stability and dynamics of CCMV virions. The discussion section addresses the

290   significance of these observations.

291

292   **Influence of viral replicase on the stability and capsid dynamics.** Analysis of trypsin

293   digestion profiles of BMV (9) and CCMV (Fig. 5; Table 1) revealed a minor but clear

294   difference in the capsid dynamics of the respective viruses. Despite variation in the

295   genome sequence (15), construction of the stable hybrid viruses between BMV and

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296 CCMV involving the exchange of genomic RNA3 but not RNAs 1 and 2 have been

297 reported (15). *N. benthamiana* leaves were infiltrated with the desired mixture of the

298 agro-inocula resulting in the assembly of two sets of hybrid viruses (Fig. 7A) to verify the

299 effect of the heterologous replicase on the dynamics of the assembled hybrid virions.

300 The first set of hybrid viruses involved the following combination of the inocula:

301 pB1+pB2+pC3 or pC1+pC2+pB3. These inocula would result in the assembly of virions

302 using either CCMV CP (i.e., CCMV virions packaging BMV RNAs 1 and 2 and CCMV

303 RNA 3+4) or BMV CP (BMV virions packaging CCMV RNAs 1 and 2 and BMV RNA

304 3+4).

305   Genomic RNA3 is amenable for exchanging between BMV and CCMV (15).

306 Therefore, we considered the assembly hybrid virions of RNAs 3+4 under heterologous

307 replicase. Consequently, infiltration of pB1a+pB2a+pC3 would result in the assembly of

308 C3+4^V by the CCMV CP generated by the BMV replicase. Likewise, infiltration of

309 pC1a+pC2a+pB3 would result in the assembly of B3+4^V by the BMV CP generated by

310 the CCMV replicase (Fig. 7A). Infiltration of the inocula resulting in the assembly of

311 respective Wt viruses (BMV and CCMV) or a virion type packaging RNA 3+4 using

312 homologous replicase (Fig. 7A; e.g., pB1+pB2+pB3; pC1+pC2+pC3 and

313 pB1a+pB2a+pB3 and pC1a+pC2a+pC3) served as controls. Western blot analysis and

314 electrophoretic mobility pattern (Fig. 7 B, C) confirmed the hybrid nature of the purified

315 virions assembled under the direction of homologous and heterologous replicases.

316   The DSF analysis (Fig. 7 D, E) of the temperature-dependent melting of the two

317 hybrid viruses and control samples (Wt BMV and Wt CCMV) was carried out under an

318 environment that closely mimics the pH of living cells (phosphate buffer, pH 7.2).

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319   Virions of BMV and CCMV assembled in the presence of homologous replicase (i.e.,

320   B1+B2+B3 and C1+C2+C3) showed distinct thermal denaturation profiles. For example,

321   most BMV virions melted at 49°C while a small population at 85°C (Fig. 7D; black

322   peaks). By contrast, CCMV virions displayed two nearly equal populations: one melting

323   at 45°C and the other at 79°C (Fig. 7E; black peaks). The role of viral replicase on the

324   thermal profiles is evident when each hybrid virus was subjected to DSF analysis.

325   Assembly of virions by the BMV CP synthesized by the heterologous replicase

326   (C1+C2+B3; Fig. 7D) mimicked the melting profiles of Wt CCMV (C1+C2+C3; Fig. 7E).

327   However, virions assembled with CCMV CP generated by the heterologous replicase

328   (B1+B2+C3; Fig. 7E) appeared different in their thermal profiles compared to that of Wt

329   BMV (B1+B2+B3; Fig. 7D). This variation perhaps can be attributed to the

330   disproportionate accumulation of all three virion types.

331   The effect of heterologous replicase on the stability of virions is evident when the

332   thermal profiles of a single virion type (i.e., B3+4^V or C3+4^V), assembled with

333   homologous vs. heterologous replicase, was comparatively analyzed (Fig. 7F, G). For

334   example, the melting profile of B3+4^V virions resembled those of Wt BMV virions, i.e.,

335   displaying a significant peak at 49°C and a minor one at 85°C (compare black and blue

336   peaks in Fig. 7D and F, respectively). By contrast, the melting profile of B3+4^V virions

337   assembled with the heterologous replicase displayed two prominent peaks: one at 59°C

338   and another at 88°C (Fig. 7F, red peaks). Likewise, the melting profile of C3+4^V virions

339   assembled with homologous replicase is distinct from that of heterologous replicase

340   (Fig. 7 E, G).

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341 Next, we tested the sensitivity of B3+4^V or C3+4^V virions assembled under

342 heterologous replicase to trypsin digestion. Compared to B3+4^V virions assembled

343 under the homologous replicase (i.e., p1a+p2a+B3; Fig. 8A, lane 1), a majority of the

344 virions assembled under the heterologous replicase remained resistant to trypsin

345 digestion (i.e., pC1a+pC2a+B3; Fig. 8A, lane 2). By contrast, a majority (~80%) of

346 C3+4^V virions assembled under homologous replicase remained intact (i.e.,

347 pC1a+pC2a+C3) while those assembled under heterologous replicase (i.e.,

348 p1a+p2a+C3) remained completely resistant (Fig. 8A bottom panel, lane 3)

349 indistinguishable from the undigested control (Fig. 8 top panel, lane 3). EM analysis

350 further substantiated these observations (Fig. 8B).

351 Finally, because of its high sensitivity, MALDI-TOF was used to identify the

352 cleavage products released due to trypsin digestion for B3+4^V or C3+4^V assembled

353 under homologous and heterologous replicases (Fig. 9). Analysis of the released

354 peptides at early time points (10 min) distinguished B3+4^V and C3+4^V virions assembled

355 with homologous replicase-CP interaction vs. heterologous replicase-CP interaction. In

356 agreement with the previous report (9), the surface of B3+4^V virions assembled with the

357 homologous BMV replicase exposes K65, R103, K111, and K165 residues (Fig. 9A;

358 Table 2). In these virions, although peptides representing N-terminal regions (21 to 26

359 aa) were detected at the earliest time point (e.g., 10 min) (Fig. 9), their accessibility to

360 protease coincided with other cleavage sites as well (Fig. 9; Table 1). Therefore, it is

361 likely that these N-terminal peptides on virions of B3+4^V may not be the first sites of

362 protease cleavage since multiple simultaneous cleavages at other sites expose the

363 otherwise internalized N-terminal region to proteolysis. Detection of peptides

16

364 encompassing the N-proximal 21 to 26 and 20 to 26 aa region at an early time point

365 (10 min) suggests their externalization in B3+4^V (Fig. 9). Alternatively, it is likely that the

366 extensive cleavage of the capsid as early as 10 min by trypsin (Fig. 9) results in

367 degradation of the capsid structure (Fig. 7B), which leads to externalization of the N

368 terminus and its subsequent proteolysis.

369   In comparison, virions of B3+4^V assembled with heterologous replicase (i.e.,

370 pC1a+ pC2a+ pB3; Fig. 9B) did not release peptides encompassing previously reported

371 surface residues (K65, K111, and K165) indicating these residues are internalized and

372 not accessible to trypsin cleavage. Also, these virions exposed their N-ARM on the

373 capsid surface, as evidenced by the cleavage products recovered (Fig. 9B; Table 2).

374 However, these residues are not a part of the structure. Therefore, any disruption

375 encompassing them did not impact the overall structural integrity of the virions (Fig. 8B).

376 Table 2 summarizes a detailed profile of the peptide fragments released following

377 trypsin digestion of B3+4^V virions assembled under homologs vs. heterologous

378 replicase.

379   The MALDI profile of trypsin-digested C3+4^V assembled under homologous

380 replicase (Fig. 9C) mirror the profile shown in Fig. 5C. Interestingly, the integrity of

381 C3+4^V virions assembled under heterologous replicase (Fig. 9D) confirms the release of

382 only negligible amounts of fragments encompassing the N-terminus (Table 3). We

383 presume that a relatively small subset of virions is involved in the release of the low

384 abundance of fragments like 66-82, 91-106, and 131-132.

385

17

Discussion

Despite being similar in the virion structure, genome organization, and replication strategies, CCMV and BMV are biologically distinct: CCMV is adapted to cowpea, a dicot host, while BMV to barley, a monocot host (18). Additionally, both CCMV and BMV also differ in the form of the CP (in conjunction with MP) required for mediating cell-to-cell and long-distance movement (20, 21). Based on this intimacy of CP to host-related activities, we hypothesized that otherwise concealed local and global dynamical conformational changes of the viral capsids are likely to contribute to the host specificity mentioned above. In this study, we extended the agroinfiltration approach (9) to assemble the three virion types of CCMV, $C1^V$, $C2^V$, and $C3+4^V$ (Fig. 1). We analyzed each virions type's relative stability and capsid dynamics with the application of thermal denaturation analysis followed by peptide-based MALDI-TOF. Subsequently, we evaluated how the type of the host plant and the viral replicase contribute toward modulation of the capsid dynamics. When compared to the previously characterized capsid dynamics of the three virions types of BMV (i.e., $B1^V$, $B2^V$, and $B3+4^V$) to those of CCMV, the results of this study revealed the following: (i) virions of $C1^V$ and $C2^V$ are indistinguishable in thermal stability and capsid dynamics; (ii) $C1^V$ and $C2^V$ are dynamically very similar to $B1^V$ and $B2^V$; (iii) capsid dynamics of $C3+4^V$ are different from those of $C1^V$ and $C2^V$, and $B3+4^V$; (iv) the type of host organism had a profound influence on the capsid dynamics of Wt CCMV virions, and finally, (v) virions of $B3+4^V$ and $C3+^{4V}$ assembled with heterologous viral replicase noticeably altered the dynamics. Below we discuss the significance of these observations in the context of bromovirus biology.

409

Comparative thermal stability and capsid dynamics of the three virion types of CCMV and BMV. Previous biochemical and biophysical studies performed in the last three decades showed that purified virions of Wt CCMV (i.e., a mixture of C1^V, C2^V, and C3+4^V) are morphologically homogeneous and are challenging to fractionate into three distinct virions types (17). Bromoviruses are known to be highly stable at the experimental pH of 4.5 (18). Therefore, at pH 4.5, all three virions types of CCMV did not reveal any difference in their thermal stability (Fig. 3A). However, viral capsid must be flexible to release the packaged genome into the cell to initiate replication followed by infection. Consequently, at pH 7, a physiological pH of the plant cell, bromoviruses have been shown to dissociate into dimers (19). DSF analysis at pH 7.2 revealed variations in the thermal stability of three virion types of CCMV (Fig. 3 A, B). Therefore, the varied instability of C1^V/C2^V vs. C+4^V (Fig. 3, A and B) in conjunction with altered capsid dynamics are implicated in the infection process (see below).

Virion types of C1^V/C2^V and B1^V/B2^V are dynamically similar: Concerning trypsin sensitivity, virion types 1 and 2 (i.e., C1^V/C2^V and B1^V/B2^V) did not reveal any clear distinction. For example, as observed with B1^V and B2^V (9), trypsin digestion did not affect the integrity of C1^V and C2^V virions (Fig. 4A, B) despite the release of 2-15 amino acids encompassing the N-terminal proximal region (Fig. 5 F-G; Table 1). It is not surprising since, in CCMV and BMV, the first N-proximal 25 amino acid region is not visible in the crystal structure and is internalized by interacting with RNA during encapsidation (20, 21). Therefore, trypsin cleavage followed by MALDI-TOF analysis

431   suggested that the capsid surface transiently exposes the N-proximal region, and its

432   release did not affect the integrity of the $C1^V$ and $C2^V$. Therefore, since the genomic

433   RNAs encapsidated by $C1^V$ and $C2^V$ encode replicase proteins, the transient exposure

434   of the N-proximal region of $C1^V$ and $C2^V$ is linked to the co-translational disassembly

435   mechanism, as previously hypothesized for $B1^V$ and $B2^V$ (9).

436   *(i)*   *Virion types* of *$C3+4^V$ and $B3+4^V$ are dynamically distinct:* A close

437   comparison of trypsin sensitivity profiles of virions of $C3+4^V$ and $B3+4^V$ displayed a

438   scenario contrasting to that of $C1^V/C2^V$ and $B1^V/B2^V$. The partial and complete

439   disruption of virion integrity for $C3+4^V$ (Fig. 4A, B) and $B3+4^V$ (9) support these

440   observations. In bromoviruses, virion assembly requires dimerization of the N- and C-

441   terminal CP subunits, and the C-terminal region plays a critical role in this process (16).

442   Consequently, exposure of the C-terminal region to trypsin as early as 10 min is

443   sufficient to abolish the virion integrity. However, a significant difference between the

444     $C3+4^V$ vs. $B3+4^V$ is the relative accessibility of the C-terminus for trypsin digestion. For

445   example, since a significant percentage of virions remain intact (Fig. 4B) suggests that

446   in $C3+4^V$, only a certain percentage of virions expose the C-proximal region for trypsin

447   digestion. Therefore, it is reasonable to assume the segregation of $C3+4^V$ virions into

448   two distinct populations. A major population is resistant to trypsin (due to inaccessibility

449   of the critical C-terminal peptides involved in the CP dimerization), and a minor

450   population is susceptible to trypsin (due to transient exposure of the C-terminal

451   peptides). By contrast, following trypsin digestion, the integrity of the entire population of

452     $B3+4^V$ virions was completely disrupted (9).

453   *Then, how do these two contrasting scenarios of C3+4^V vs. B3+4^V contribute to*

454   *the life cycles of CCMV and BMV?* We offer the following explanation. In bromoviruses,

455   gene products encoded by RNA3 play essential roles in replication and host adaptability

456   (22). In CCMV and BMV, CP mRNA production depends on the replication of the

457   genomic RNA3 (4, 23). Following translation, CP stimulates the asymmetric synthesis of

458   the plus-strand over the minus strand (24), and its interaction with replicase dictates

459   packaging specificity (25). Therefore, based on the capsid dynamics of B3+4^V, we

460   previously hypothesized that the complete release of the genomic RNA3 is obligatory

461   for its replication (9). However, this study revealed that, unlike B3+4^V, most C3+4^V

462   virions are stable. Taken together, we speculate that virions of C3+4^V and B3+4^V

463   evolved to modulate their capsid dynamics during *in vivo* assembly to adapt to their

464   respective natural hosts and the CP form required for the cell-to-cell movement.

465   **Modulation of the capsid dynamics by the host organism.** Studies analyzing the

466   capsid dynamics of RNA viruses pathogenic to animals and plants offer new insight into

467   how the capsid dynamics fluctuations would control the overall infectivity and

468   pathogenesis (26). Results of this study analyzing the capsid dynamics of CCMV virions

469   obtained from cowpea and *N. benthamiana* host plants (Fig. 6 A-D) accentuates the

470   significance of the viral capsid's flexibility in establishing a successful infection. Viral

471   infection in a given host plant involves two active processes. These are cell-to-cell and

472   long-distance movement. Following entry and initial replication in primary cells, all plant

473   viruses must spread to the neighboring cells (i.e., cell-to-cell movement) via

474   plasmodesmata with the help of a dedicated and virus-encoded MP (27). However, in

475   BMV and CCMV, both MP and CP are obligatory for cell-to-cell spread (4). A majority of

476   plant viruses, including bromoviruses, manifesting the long-distance spread require

477   assembled virions. These observations suggest that an optimal interaction between the

478   host machinery and the virion surface configuration (arrangement of amino acids) is

479   necessary to mediate the long-distance spread. A change in the virion surface

480   configuration due to a mutation may lead to altered spread and pathogenesis. For

481   example, BMV induces chlorotic local lesions on inoculated leaves followed by systemic

482   mottling symptom phenotype in *Chenopodium quinoa* (10). A small deletion in the N-

483   proximal region significantly altered the lesion phenotype and blocked the systemic

484   spread (10). Given the variation in the cell types of cowpea and *N. benthamiana*,

485   considerable flexibility in capsid dynamics is envisioned for CCMV. This required

486   flexibility perhaps explains why the capsid dynamics of CCMV are distinct in cowpea

487   and *N. benthamiana* plants (Fig. 6).

488

489   **Modulation of capsid dynamics by the viral replicase.** In addition to catalyzing

490   replication, viral replicase is intimately involved in regulating other biological processes

491   such as pathogenesis and genome packaging. In BMV and CCMV, the assembly of

492   biologically viable hybrids involves exchanging genomic RNA3 (i.e., C1+C2+B3 and

493   B1+B2+C3), but not either genomic RNA 1 or RNA 2 (e.g., C1+B2+C3; B1+C2+C3)

494   (15). Furthermore, the interaction of viral replicase with the CP alters the RNA

495   packaging specificity (25). Results of this study have identified another critical role

496   played by the viral replicase, i.e., modulation of capsid dynamics reflecting the stability.

497   The effect of viral replicase on the stability and capsid dynamics became apparent since

22

498   virion types of BMV and CCMV assembled under the direction of homologous and

499   heterologous replicase displayed contrasting profiles (Figs. 7-9; Tables 2 and 3).

500   Based on the MALDI-TOF data (Tables 1-3), how the dynamics of B3+4^V and

501   C3+4^V virions are modulated by the homologous and heterologous replicase is

502   schematically shown in Fig.10. Linear CP of BMV and CCMV respectively expose

503   approximately 24 and 30 trypsin cleavage sites [Fig. 10, Panels A, B (i)]. In the case of

504   BMV, the interaction of the CP subunits with the homologous replicase results in the

505   assembly of B3+4^V virions with a confirmation presenting 15 of the 24 trypsin cleavage

506   sites on the surface [Fig. 10, Panel A (ii)]. This conformation rendered B3+4^V virions

507   highly susceptible to trypsin and affected the structural integrity (Fig. 8A, lane 1). By

508   contrast, the assembly of B3+4^V virions under heterologous replicase altered the

509   surface conformation presenting 10 of the 24 trypsin cleavage sites on the surface [Fig.

510   10, Panel A (iii)]. This configuration rendered B3+4^V virions resistant to trypsin (Fig. 8,

511   lane 2).

512   With respect to CCMV, the interaction of the CCMV CP subunits with the

513   homologous replicase results in the assembly of C3+4^V virions with a confirmation

514   presenting approximately 24 of the 30 trypsin cleavage sites on the surface [Fig. 10,

515   Panel B (ii)]. This confirmation rendered ~20% of C3+4^V virions susceptible to trypsin

516   while the remaining 80% remained structurally intact (Fig. 8). Whereas C3+4^V virions

517   assembled under the heterologous replicase positioned 10 of the 30 trypsin cleavage

518   sites and rendered 100% virions resistance to trypsin [Fig. 10, Panel B (iii)]. The

519   contrasting trypsin sensitivity of B3+4^V and C3+4^V virions assembled under homologous

520   vs. heterologous replicase (Figs. 8-10) provides compelling evidence for the intimate

521 involvement of the viral replicase in modulating the capsid dynamics. Apparently,

522 interaction with the viral replicase positions the biologically relevant peptides on the

523 capsid surface for optimal interaction with the host for successful onset of the infection.

524 These observations explain why neither hybrid virus (i.e., C1+C2+B3 or B1+B2+C3)

525 systemically infected the natural host of CCMV and BMV (15).

526   In conclusion, this study accentuates previously unrecognized structural

527 differences among the three virions of CCMV and BMV and their importance in the

528 general biology of the two viruses. The previously determined cryo-structure of Wt

529 CCMV represents the average of three virions (20). Therefore, the strategy used in this

530 study (Fig. 1) would be ideal for comparatively assessing the structure of independent

531 virion types. Such studies are likely to shed more light on the structural and

532 conformational heterogeneity of the three virions types and their role in viral biology and

533 pathogenesis.

534

535 **Materials and Methods**

536

537   **Agro-plasmids used in this study.** The construction and characteristic

538 properties of agro-plasmids pC1, pC2, and pC3 (Fig. 1, Panel IA), engineered to

539 express biologically active full-length genomic RNAs of CCMV following agroinfiltration

540 into plants, are described previously (9). Likewise, agro-plasmids pC1a, pC2a, and

541 pCCP (Fig. 1, Panel IB) are engineered respectively to transiently express replicase

24

542 protein1a (p1a), replicase protein 2a (p2a) and CP are as described previously (9).

543 Agro constructs for expressing the full-length BMV genomic RNAs (Fig. 1, Panel IC) and

544 for transiently expressing the replicase, and CP (Fig. 1, Panel ID) are previously

545 described.

546

547 **Virion purification, electron microscopy (EM), virion RNA electrophoresis,**

548 **and Western blot analysis.** The procedure used to purify CCMV and BMV virions from

549 4-6 days post-infiltration (dpi) agroinfiltrated leaves or mechanically inoculated cowpea

550 (for CCMV) and *N. benthamiana* (both CCMV and BMV) was as described by Rao et al.

551 (28). For negative-stain EM analysis, gradient purified virions were spread on glow-

552 discharged grids followed by negative-staining with 2% uranyl acetate prior to

553 examination with a Technai 12 transmission electron microscope operated at 120KeV

554 (Center for Advanced Microscopy and Microanalysis facility at UC-Riverside) and

555 images were recorded digitally. For electrophoretic mobility analysis, purified virions

556 were loaded in a 1% agarose gel prepared and electrophoresed in virus suspension

557 buffer at 7v/cm for 2-2.5 hr at 4^0C. Western blot analysis of un-digested and trypsin-

558 digested virion samples using the desired anti-CP (either anti-CCMV CP or a mixture of

559 both anti-BMV CP and anti-CCMV CP) was performed as described previously (29).

560

561 **Differential scanning fluorimetry (DSF).** DSF was performed essentially as

562 described previously (30). Desired virus and control (lysozyme) samples were re-

563 suspended either in nano pure sterile water (pH 7.1) or in virus suspension buffer (50

564 mM sodium acetate, 8 mM magnesium acetate, pH 4.5) or in 100 mM Phosphate buffer

565 (pH 7.2). The experiment was performed three times independently using three

566 replicates for each sample. DSF data were analyzed as described by Rayaprolu *et al*.

567 (30) and plotted using GraphPad Prism version 9.3.1 for macOS, GraphPad Software,

568 San Diego, California USA, www.graphpad.com.

569

570 **MALDI-TOF.** For MALDI-TOF analysis, a desired purified virion preparation was

571 diluted to 1 mg/ml in 25 mM Tris-HCl, 1 mM EDTA buffer. A 30 µl sample (i.e., 30 µg of

572 the virus) was digested with 1:100 (w/w) ratios of trypsin (Pierce Trypsin Protease mass

573 spectrometry grade; Thermo Fisher Scientific)/virus for various time points at 25°C (31,

574 32). A 20 µl of water was added to 10 µl of the digested sample, and approximately 0.5

575 µl of each digest or undigested (control) sample was analyzed on AB Sciex TOF/TOF

576 5800 MALDI MS with a-cyano-4-hydroxycinnamic acid matrix. The instrument was

577 calibrated with standards, and the test samples were analyzed with external calibration.

578 The accuracy is about +/- 0.05 dalton. The MALDI-TOF data were analyzed with AB

579 Sciex Data Explorer, and baseline was corrected with peak width 32, flexibility 0.5 and

580 degree 0.1. For noise removal, the standard deviation was set at 2. To determine the

581 peak detection criteria, % centroid was taken as 50, signal/noise (S/N) threshold was 3,

582 and the noise window width m/z was 250. The threshold after S/N recalculation was set

583 at 20. The peptide fragments were assigned based on the UCSF Protein Prospector's

584 MS-Digest function (33).

585

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594

595

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680

681   **FIGURE LEGENDS**

31

682

FIG 1 Agroplasmids and agroinfiltration *in planta*. *Panel I*. (A) Characteristics of T-DNA-based agroplasmids for the transient expression of full-length CCMV genomic RNAs in plants as described previously for BMV (9). The 35S-C1 (pC1), 35S-C2 (pC2), and 35S-C3 (pC3) constructs respectively contain the full-length cDNA copies of CCMV genomic RNA1 (C1), -2 (C2), and -3 (C3). Single lines and solid boxes represent noncoding and coding regions, respectively. Monocistronic C1 encoding replicase protein 1a (C1a) and monocistronic C2 encoding replicase protein 2a (C2a) are indicated. In C3, the locations of the movement protein (CMP) and capsid protein (CCP) genes are shown. At the 3' end of each construct, the clover leaf-like structure represents a tRNA-like motif conserved among all three CCMV genomic RNAs. A set of two black arrows at the 5' ends represent the double 35-S promoter. A bent arrow indicates the predicted self-cleavage site by the ribozyme. The location of the *Nos* terminator is indicated. (B) Agroconstructs for transient expression of C1a, C2a, and CP (pCCP). Open reading frames (ORFs) of CCMV p1a, p2a, and CP were fused in-frame to a binary vector using *Stu*I and *Spe*I sites. (C) Characteristics of agroplasmids for the transient expression of full-length BMV genomic RNAs in plants as described previously (9). (D) Features agroconstructs for the transient expression of BMV replicase proteins (B1a and B2a), and CP (pBCP) as described previously (9). *Panel II*. Agroinfiltration strategy used for the assembly of each virion type of CCMV. (A) (Positive control) Infiltration of the inoculum containing a mixture of pC1+pC2+pC3 would result in the induction of Wt CCMV infection containing a mixture of all three virions (i.e., $C1^V$, $C2^V$, $C3+4^V$). (B) (Negative control) Infiltration of the inoculum containing agroplasmid pCCP would result

705   in the expression of CP mRNA for the translation of Wt CP and its non-specific

706   assembly of virions containing CP mRNA and cellular RNA. (C) Assembly of virions

707   packaging CCMV RNA1 (C1^V). A mixture of inoculum containing agrotransformants

708   pC1+pC2a+pCCP (see Fig. 1 panel I for details) is infiltrated into *N. benthamiana*

709   leaves to result the following. (i) Transcription of pC1 results in the synthesis of a

710   biologically active full-length genomic RNA1 for the translation of functional replicase

711   protein 1a; (ii) agrotransformant pC2a results in an mRNA competent for the translation

712   of replicase 2a but not replication because it lacks the 5' and 3' noncoding regions and

713   finally (iii) translation of the mRNA transcribed from agrotransformant pCCP gives the

714   CP subunits for directing virion assembly. Assembly of functional replicase results from

715   the interaction of proteins 1a and 2a catalyzes the replication of C1 RNA, followed by its

716   packaging into virions by the transiently expressed CCMV CP subunits. (D) Assembly of

717   virions packaging CCMV RNA2 (C2^V). The inoculum shown in this panel is identical to

718   the one shown in panel C, except that pC1 and pC2a are replaced by pC1a and pC2,

719   respectively. Agroinfiltration of this inoculum results in the assembly of virions

720   containing C2 RNA. (E) Assembly of virions packaging CCMV RNA3 and -4 (C3+4^V).

721   The inoculum shown in this panel is formulated to assemble virions packaging C3 RNA

722   and sgC4 by the infiltration of a mixture of pC1a, pC2a, and pC3 agrotransformants.

723   Transiently expressed replicase proteins 1a and 2a directs the replication of C3,

724   followed by the synthesis of sgC4 for CP production. Fig1 panel II was created with

725   BioRender.com.

726

727   **FIG 2** Progeny analysis of the three individually assembled virion types of CCMV (C1^V,

728   C2^V, and C3+4^V). *(A) Western blot analysis.* Total protein preparations from *N.*

729   *benthamiana* leaves at 4 dpi were extracted and analyzed using an anti-CCMV CP

730   antibody. *Lane 1*, pC1+pC2a+pCCP resulting in the assembly of C1^V; *Lane 2,*

731   pC1a+pC2+pCCP resulting in the assembly of C2^V; *Lane 3,* pC1a+pC2a+pC3 resulting

732   in the assembly of C3+4^V; *Lane 4*, pCCP resulting in the assembly CCP^V; *Lane 5*,

733   pC1+pC2+pC3 resulting in the assembly of Wt CCMV infection (i.e., a mixture of C1^V,

734   C2^V, and C3+4^V). In each lane, a sample containing 30 µg of total leaf protein

735   (estimated by the Bradford protein assay) was loaded. *(B) EM analysis.* Electron

736   micrographs of the density gradient-purified virions of Wt CCMV from either *N.*

737   *benthamiana* or cowpea plants or autonomously assembled virions of C1^V, C2^V, and

738   C3+4^V via agroinfiltration. Virion samples were negatively stained with uranyl acetate

739   prior to examining under the EM. The scale bar is indicated on each image. *(C) Virion*

740   *electrophoresis.* Density gradient-purified virions of Wt BMV (lane 1), Wt CCMV (lane

741   2), C1^V (lane 3), C2^V (lane 4), and C3+4^V (lane 5) were subjected to electrophoresis in

742   an agarose gel as described under Materials and Methods. The gel shown on the top

743   panel was stained with ethidium bromide to detect RNA and then re-stained with

744   Coomassie Brilliant Blue R-250 to detect the protein (bottom panel). The positions of

745   BMV and CCMV virions migrating toward negative and positive, respectively, are

746   indicated.

747

748   **FIG 3** Stability analysis of the three CCMV virion types using differential scanning

749   fluorimetry (DSF). DSF measurements of derivative of fluorescence intensity as a

750 function of temperature for each virion type normalized so that the maximum of the

751 derivative is set to 1 (y-axis) during heating of (A and B) $C1^V$, $C2^V$, and $C3+4^V$ and (C

752 and D) lysozyme (control) at various temperatures (x-axis) under indicated conditions.

753 Panel A: Virions of $C1^V$, $C2^V$, and $C3+4^V$ displayed identical temperature dependence

754 (~70°C) for melting under virus suspension buffer conditions at pH 4.5. Panel B: At pH

755 7.2, a portion of virions of $C1^V$, $C2^V$, and $C3+4^V$ melted at lower temperatures (~50°C),

756 whereas $C1^V$ and $C2^V$ displayed another major thermally unstable population of peaks

757 at ~90°C (see text for details). Panel C and D: Lysozyme used as positive control

758 showed identical melting characteristics (~70°C) under both pH conditions. The figures

759 indicate representative data from N=3 independent experiments.

760

761

762 **FIG 4** Proteolysis of $C1^V$, $C2^V$, $C3+4^V$, CCP^V. *(A) Western blot analysis*: Virion

763 preparations of either $C1^V$ or $C2^V$ or $C3+4^V$ or CCP^V were either undigested or digested

764 with trypsin for 10 min, 30 min, or 45 min and subjected to Western blot analysis as

765 described under the Materials and Methods section. *(B) EM analysis:* Negative-stain

766 electron micrographs showing the integrity of the undigested and trypsin-digested virion

767 preparations of the indicated samples. The scale bar is indicated on each image.

768

769 **FIG 5** The organization of the three CP subunits (A, B, and C) on the surface of the

770 CCMV virions and analysis of the peptides released by the trypsin digestion of virions.

771 (A and B): Structure of CCMV capsid based on the PDB entry 1CWP showing the A, B,

772 C subunits and T=3 lattice. (C-E): The position of the trypsin cleavage sites located on

35

773   each CP subunit A, B, and C. (F-H) MALDI-TOF analysis of peptides released from

774   virions of either C1^V or C2^V or C3+4^V following digestion with trypsin at the indicated

775   time point. Peaks are labeled with corresponding polypeptide fragments of predicted

776   amino acid residues. Table 1 summarizes masses and identifies the corresponding

777   amino acid residues.

778

779   **FIG 6** Effect of the host on the stability of Wt CCMV virions. (A) Stability analysis of the

780   Wt CCMV virions isolated from either cowpea or *N. benthamiana* leaves using

781   differential scanning fluorimetry (DSF). (Left panel) Most of the Wt CCMV virions

782   purified from both host plants displayed a single peak melting at ~70°C at pH 4.5. (Right

783   panel) At pH 7.2, CCMV virions purified from cowpea displayed a major peak at ~50°C

784   and a minor peak at ~90°C, whereas CCMV virions obtained from *N. benthamiana*

785   plants displayed two major peaks with equal intensity, one at ~50°C and the other at

786   ~80°C. (B) Western blot analysis of Wt CCMV from cowpea and *N. benthamiana* (NB).

787   Prior to Western blot analysis, each virion preparation was either undigested or digested

788   with trypsin for 10 min, 30 min, or 45 min. (C) Negative-stain electron micrographs show

789   the integrity of the undigested and trypsin-digested virion preparations of Wt CCMV

790   from cowpea and *N. benthamiana* (NB). (D) MALDI-TOF analysis. Wt CCMV virions

791   from cowpea and *N. benthamiana* are subjected to MALDI-TOF analysis following

792   trypsin digestion for 10 min as described in the Methods section. Peaks are labeled with

793   corresponding polypeptide fragments of predicted amino acid residues.

794

795 **FIG 7** Properties of Wild-type and hybrid viruses constructed between BMV and CCMV.

796 (A) Schematic representation of the strategy used for the (i) assembly of control virions

797 of BMV Wt (pB1+pB2+pB3) and CCMV Wt (pC1+pC2+pC3); (ii) autonomous assembly

798 of either $B1^V$ or $B2^V$ or $B3+4^V$ as described previously; (iii) autonomous assembly of

799 either $C1^V$, $C2^V$, and C3+4; (iv) hybrid virions assembled by the CCMV CP with BMV

800 heterologous replicase: pB1+pB2+pC3 and hybrid virions assembled by the BMV CP

801 with CCMV replicase: pC1+pC2+pC3; (v) assembly of virion type C3+4 with BMV

802 replicase (pB1a+pB2a+pC3) and assembly of virion type B3+4 with CCMV replicase

803 (pC1a+pC2a+pB3). (B) (Top panel) Western blot analysis. Lanes 1-8: Total protein

804 preparations extracted from *N. benthamiana* at 4 dpi infiltrated with the indicated

805 mixture of agrotransformants were subjected to Western blot analysis using anti-CP

806 antibody against CCMV and BMV. Each lane has 30 μg of total protein estimated by

807 Bradford protein assay. (Bottom panel) The gel is stained with Coomassie Brilliant Blue

808 R-250 to demonstrate the loading controls. The samples are loaded in the order as

809 mentioned above in lanes 1-8. (C) Electrophoretic mobility profiles: The following virion

810 preparations were subjected to electrophoresis as described in the Materials and

811 Methods section. Lanes 1 (left panel) Wt BMV or CCMV (right panel); Lanes 2 (left

812 panel) hybrid virions of BMV assembled with CCMV CP, or hybrid virions of CCMV

813 assembled with BMV CP (right panel); Lanes 3 left and right panel, respectively

814 represent the virion type $B3+4^V$ and $C3+4^V$ assembled with homologous replicase;

815 Lanes 4 left and right panel, respectively represent the virion type $B3+4^V$ and $C3+4^V$.

816 The gels shown on the top panel were stained with ethidium bromide to detect RNA and

817 then re-stained with Coomassie Brilliant Blue R-250 to detect protein (bottom panel).

818 BMV and CCMV virions migrating toward negative and positive, respectively, are

819 indicated. (D-G) DSF analysis: Stability analysis of the BMV and CCMV virions

820 assembled in the presence of either homologous or heterologous replicase using

821 differential scanning fluorimetry (DSF). DSF measurements of derivative of fluorescence

822 intensity as a function of temperature for each virion type normalized so that the

823 maximum of the derivative is set to 1 (y-axis) during heating of the virions indicated at

824 various temperatures (x-axis) at pH 7.2. The virions assembled in the presence of either

825 full-length RNAs or ORFs encoding heterologous replicase show unusual and increased

826 thermal stability compared to those assembled as a result of interaction between

827 homologous replicase and CP (see the text for details).

828 **FIG 8** Capsid dynamics of B3+4^V and C3+4^V virions. (A) Western blot analysis of

829 undigested (control) and trypsin digested B3+4^V and C3+4^V virions assembled in the

830 presence of either homologous (panel A, lanes 1 and 4) or heterologous replicase

831 (panel A, lanes 2 and 3). (B) Negative-stain electron micrographs showing the integrity

832 of the undigested and trypsin-digested virion preparations are shown in panel A. A size

833 bar is given on each micrograph.

834 **FIG 9** MALDI-TOF analysis of trypsin-digested hybrid virions. (A, B) MALDI-TOF

835 analysis of peptides released from B3+4^V virions assembled with homologous and

836 heterologous replicase following digestion with trypsin at 10 min. (C-D) MALDI-TOF

837 analysis of peptides released from C3+4^V virions assembled with homologous and

838 heterologous replicase following digestion with trypsin at 10 min. Peaks are labeled with

839 corresponding polypeptide fragments of predicted amino acid residues. Table 2 and 3

840 summarize masses and identifies the corresponding amino acid residues. The figures

841 indicate representative data from N=2 independent experiments.

842

843 **FIG 10** A schematic model showing the alteration of trypsin cleavage on the surface of

844 B3+4^V and C3+4^V assembled under the homologous vs. heterologous replicase (see

845 text for details).

846

847 **TABLE 1** Kinetics of trypsin cleavage sites located on C1^V, C2^V, and C3+4^V. Amino acid

848 fragments released during the early time points by C1^V, C2^V are highlighted in light grey,

849 and those released by C3+4^V are highlighted in dark grey.

850

851 **TABLE 2** Kinetics of the trypsin cleavage sites located on the Wt BMV and its hybrid

852 virions assembled in the presence of either homologous (Wt BMV and B3+4^V) or

853 heterologous replicase (C1+ C2+ B3 and C1a+ C2a+ B3). Amino acid peptides

854 fragments recovered for each virion type are indicated with "+."

855

856 **TABLE 3** Kinetics of the trypsin cleavage sites located on the Wt CCMV and its hybrid

857 virions assembled in the presence of either homologous (Wt CCMV and C3+4^V) or

858 heterologous replicase (B1+ B2+ C3 and p1a+ p2a+ C3). Amino acid peptides

859 fragments recovered for each virion type are indicated with "+."

TABLE 1 Kinetics of trypsin cleavage sites located on C1^v, C2^v, and C3+4^v

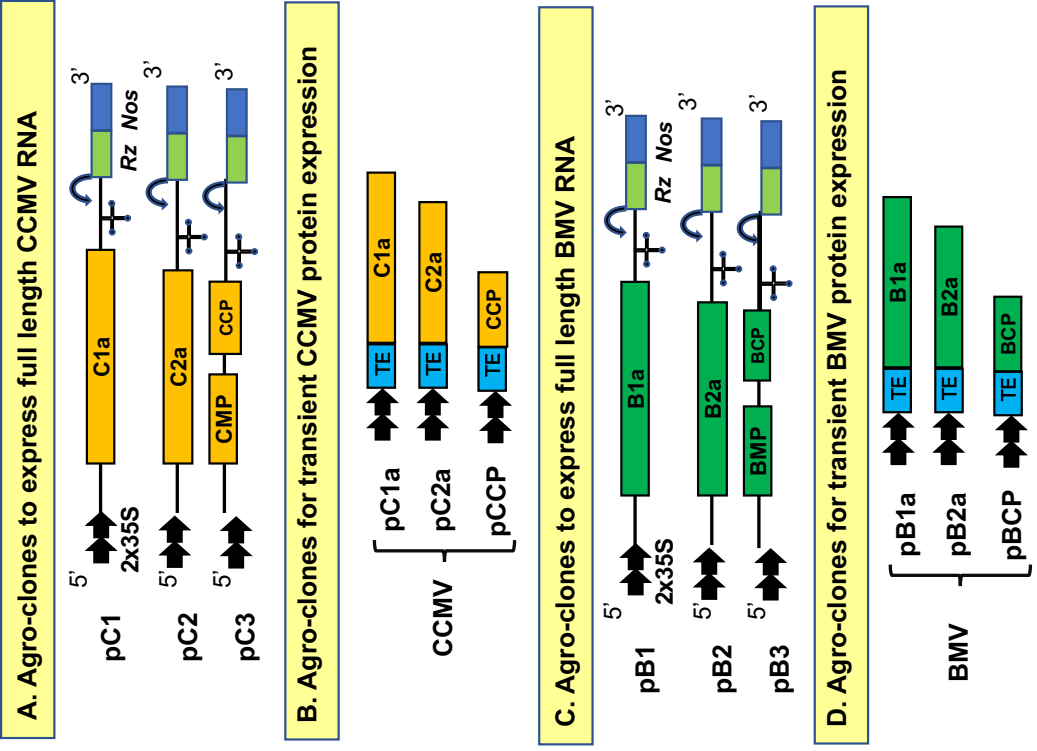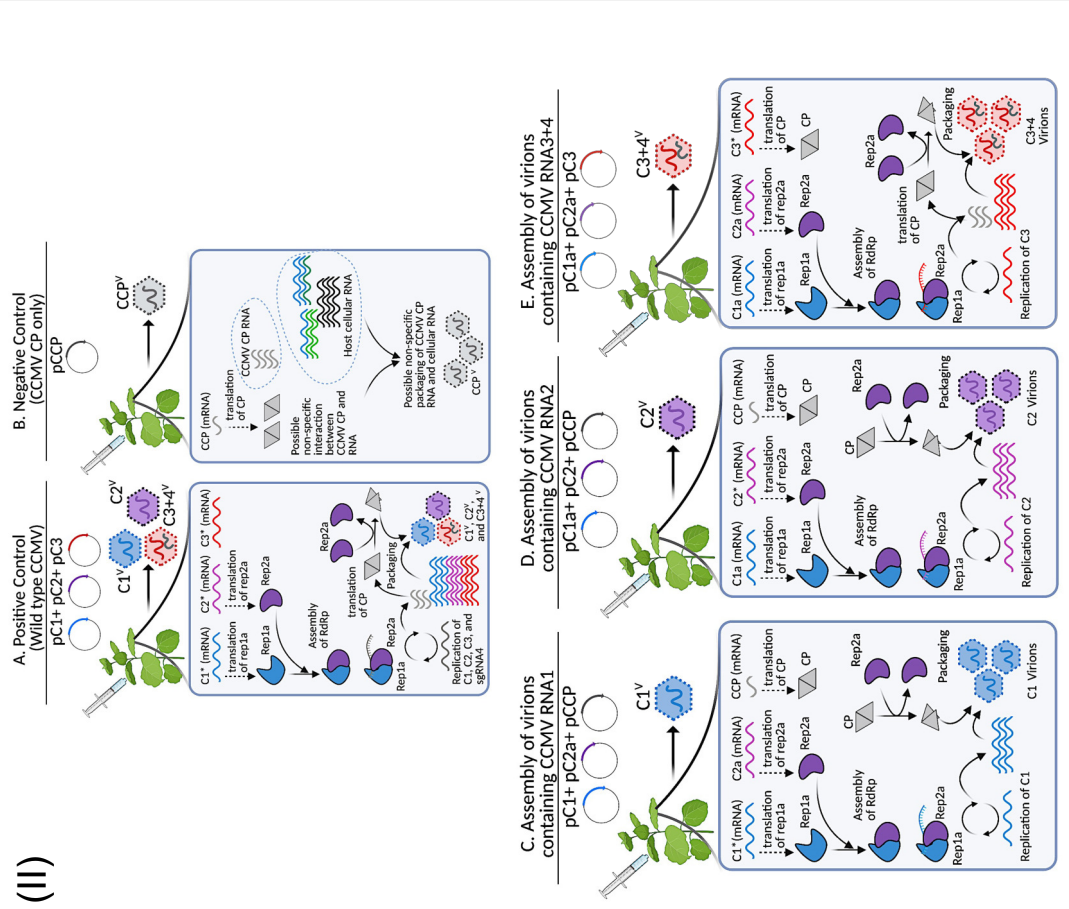|			Time to cleavage								
			C1^v			C2^v			C3+4^v		
Residues	Sequence	m/z	10 min	45 min	3 hr	10 min	45 min	3 hr	10 min	45 min	3 hr
2-11	STVGTGKLTR	1061.5949	-	-	+	+	+	+	+	+	+
2-14	STVGTGKLTRAQR	1416.7918	-	-	-	-	+	+	+	-	-
2-15	STVGTGKLTRAQRR	1572.8929	+	-	+	+	+	+	+	+	-
9-15	LTRAQRR	900.5486	-	-	-	-	-	-	+	-	-
15-20	RAAARK	672.4264	+	+	-	-	+	-	+	+	-
15-23	RAAARKNKR	1070.6654	+	+	+	-	-	-	-	-	-
21-23	NKR	417.2568	+	+	-	-	+	+	+	+	+
21-26	NKRNTR	788.4486	-	-	-	+	-	+	+	-	-
24-42	NTRVVQPVIVEPIASGQGK	1992.1237	+	-	-	-	-	-	+	+	-
24-45	NTRVVQPVIVEPIASGQGKAIK	2304.3398	-	-	+	-	-	-	+	+	-
27-42	VVQPVIVEPIASGQGK	1620.9319	-	+	-	-	+	+	+	+	+
27-45	VVQPVIVEPIASGQGKAIK	1933.1481	-	-	-	-	-	-	+	-	-
43-54	AIKAWTGYSVSK	1310.7103	-	-	-	-	-	-	+	+	+
43-65	AIKAWTGYSVSKWTASCAAAEAK	2400.2016	-	-	-	-	-	-	+	-	-
46-54	AWTGYSVSK	998.4942	-	+	+	-	-	+	+	+	+
55-65	WTASCAAAEAK	1108.5092	-	+	+	-	-	+	+	+	+
66-82	VTSAITISLPNELSSER	1816.9651	-	+	+	-	+	+	+	+	+
66-84	VTSAITISLPNELSSERNK	2059.1030	-	+	-	-	-	-	+	+	+
66-87	VTSAITISLPNELSSERNKQLK	2428.3406	-	+	-	-	-	-	+	+	+
66-90	VTSAITISLPNELSSERNKQLKVGR	2740.5316	-	-	-	-	-	-	+	-	-
83-87	NKQLK	630.3933	+	+	-	-	+	+	+	+	-
83-90	NKQLKVGR	942.5843	-	-	-	+	-	-	+	-	-
85-90	QLKVGR	700.4464	+	-	-	-	-	-	+	+	+
88-106	VGRVLLWLGLLPSVSGTVK	1994.2161	-	-	+	-	-	-	-	-	+
91-106	VLLWLGLLPSVSGTVK	1682.0251	-	+	+	-	-	-	-	-	+
107-131	SCVTETQTTAAASFQVALAVADNSK	2513.2188	-	-	+	-	-	-	+	+	+
132-143	DVVAAMYPEAFK	1340.6555	-	+	+	-	-	+	+	+	+

TABLE 2 Kinetics of trypsin cleavage sites located on Wt BMV and its hybrid virions

| | Predicted tryptic peptides of BMV CP and their masses | | Time to cleavage: 10 min | | | |
Residues	Sequence	m/z	Wt BMV	C1+C2+B3	p1a+p2a+B3	C1a+C2a+B3
2-11	STSGTGKMTR	1067.515	-	-	-	+
2-14	STSGTGKMTRAQR	1422.7118	-	-	-	+
9-15	MTRAQRR	918.505	-	+	-	+
12-20	AQRRAAARR	1055.6293	-	+	-	-
16-26	AAARRNRWTAR	1328.7407	-	+	-	-
20-26	RNRWTAR	959.5282	+	-	+	+
21-26	NRWTAR	803.4271	+	+	+	+
23-44	WTARVQPVIVEPLAAGQGKAIK	2332.35	-	-	-	+
27-41	VQPVIVEPLAAGQGK	1505.8686	-	-	+	-
27-44	VQPVIVEPLAAGQGKAIK	1818.0847	-	+	-	+
27-64	VQPVIVEPLAAGQGKAIKAIAGYSISKWEASSDAITAK	3868.1219	+	-	+	-
42-64	AIKAIAGYSISKWEASSDAITAK	2381.2711	+	-	+	-
45-64	AIAGYSISKWEASSDAITAK	2069.055	+	-	+	-
45-81	AIAGYSISKWEASSDAITAKATNAMSITLPHELSSEK	3878.9481	-	-	+	-
65-86	ATNAMSITLPHELSSEKNKELK	2441.2705	+	-	+	-
65-89	ATNAMSITLPHELSSEKNKELKVGR	2753.4614	+	-	+	-
82-86	NKELK	631.3774	-	-	-	+
82-89	NKELKVGR	943.5683	-	-	-	+
84-103	ELKVGRVLLWLGLLPSVAGR	2176.3329	-	-	+	-
87-103	VGRVLLWLGLLPSVAGR	1806.1112	+	-	+	-
90-103	VLLWLGLLPSVAGR	1493.9202	+	-	+	+
112-142	QAQAEAAFQVALAVADSSKEVVAAMYTDAFR	3258.61	+	-	+	-
131-142	EVVAAMYTDAFR	1372.6566	+	-	+	-
166-189	AVVVHLEVEHVRPTFDDFFTPVYR	2872.4781	+	-	+	-

TABLE 3 Kinetics of trypsin cleavage sites located on Wt CCMV and its hybrid virions
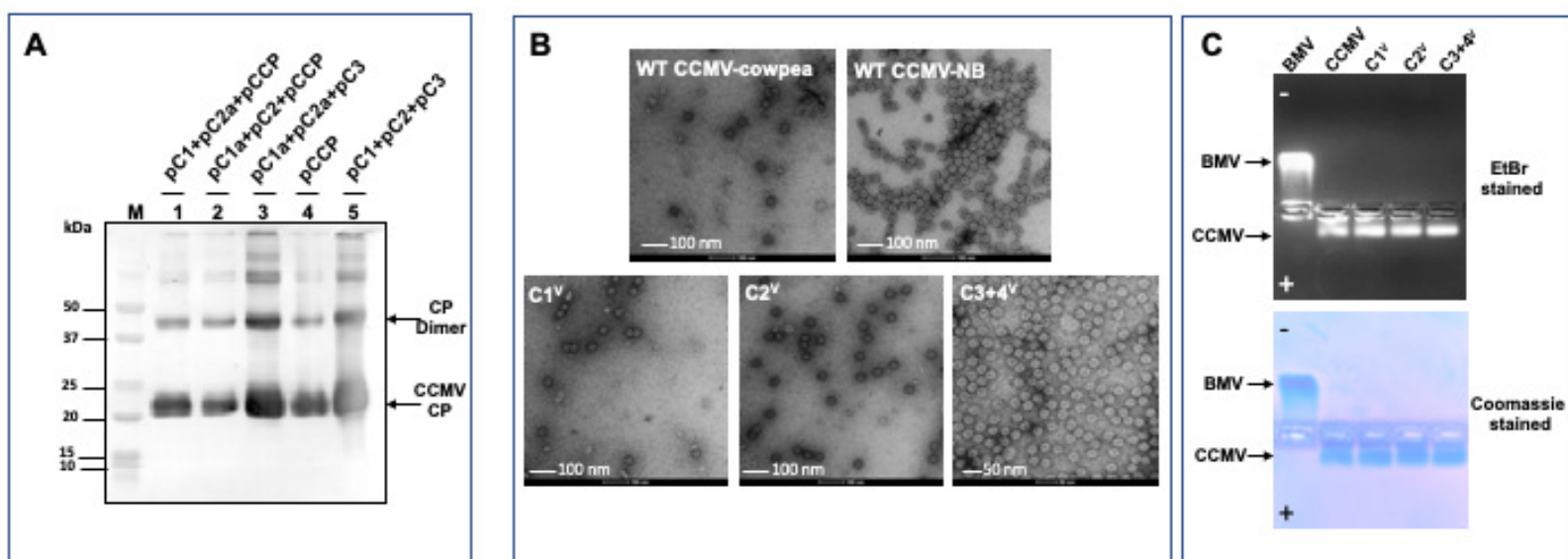
Residues	Sequence	m/z	Wt CCMV	B1+B2+C3	C1a+C2a+C3	p1a+p2a+C3
	Predicted tryptic peptides of CCMV CP and their masses			Time to cleavage: 10 min		
2-11	STVGTGKLTR	1061.5949	+	+	+	+
2-14	STVGTGKLTRAQR	1416.7918	-	-	+	+
2-15	STVGTGKLTRAQRR	1572.8929	-	-	+	+
9-11	LTR	389.2507	+	+	-	+
9-15	LTRAQRR	900.5486	-	-	-	-
15-20	RAAARK	672.4264	-	-	+	-
16-20	AAARK	516.3253	-	+	-	+
16-23	AAARKNKR	914.5643	+	-	-	-
21-23	NKR	417.2568	-	+	+	+
21-26	NKRNTR	788.4486	-	-	+	-
24-42	NTRVVQPVIVEPIASGQGK	1992.1237	-	-	+	-
24-45	NTRVVQPVIVEPIASGQGKAIK	2304.3398	-	-	+	-
27-42	VVQPVIVEPIASGQGK	1620.9319	+	+	+	+
27-45	VVQPVIVEPIASGQGKAIK	1933.1481	-	-	+	-
43-45	AIK	331.2340	+	-	-	-
43-54	AIKAWTGYSVSK	1310.7103	-	-	+	-
43-65	AIKAWTGYSVSKWTASCAAAEAK	2400.2016	-	-	+	-
46-54	AWTGYSVSK	998.4942	+	+	+	+
55-65	WTASCAAAEAK	1108.5092	+	+	+	-
66-82	VTSAITISLPNELSSER	1816.9651	+	+	+	+
66-84	VTSAITISLPNELSSERNK	2059.1030	+	+	+	-
66-87	VTSAITISLPNELSSERNKQLK	2428.3406	+	-	+	+
66-90	VTSAITISLPNELSSERNKQLKVGR	2740.5316	-	-	+	-
83-87	NKQLK	630.3933	-	+	+	-
83-90	NKQLKVGR	942.5843	-	-	+	-
85-90	QLKVGR	700.4464	-	-	+	-
88-106	VGRVLLWLGLLPSVSGTVK	1994.2161	+	-	-	-
91-106	VLLWLGLLPSVSGTVK	1682.0251	+	+	-	+
107-131	SCVTETQTTAAASFQVALAVADNSK	2513.2188	+	+	+	-
132-143	DVVAAMYPEAFK	1340.6555	+	+	+	+

A C1^V, C2^{V,}, and C3+4^V

B

C Lysozyme

D

A Subunit organization
- Subunit A (blue)
- Subunit B (red)
- Subunit C (green)

B T=3 Lattice

C Looking at residues in chain A

D Looking at residues in chain B

E Looking at residues in chain C

MALDI-TOF (10 min)

F C1^V

G C2^V

H C3+4^V
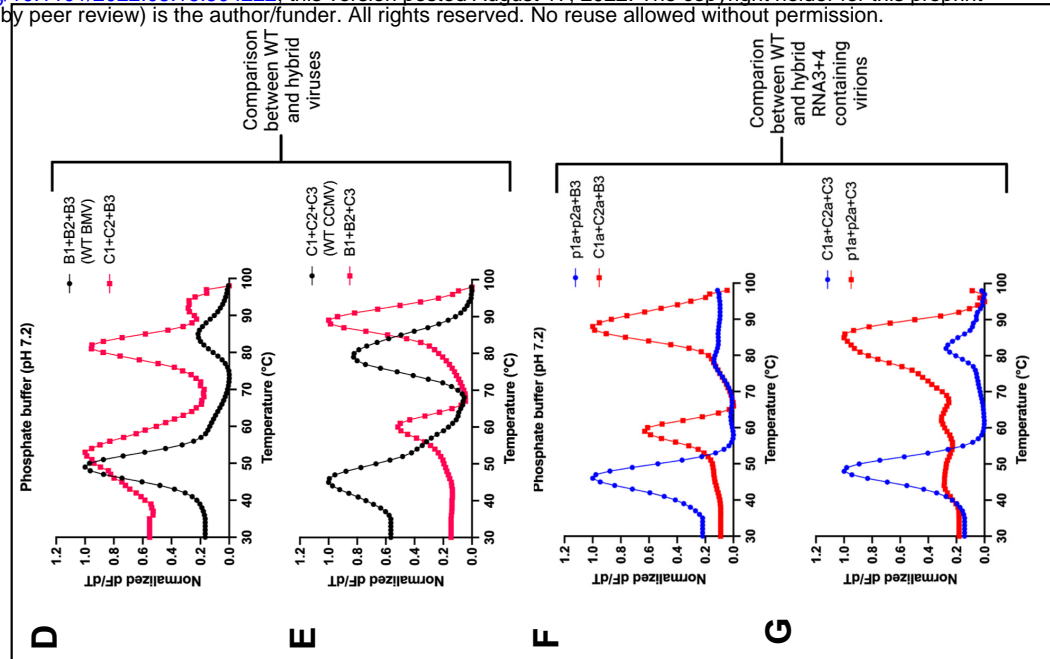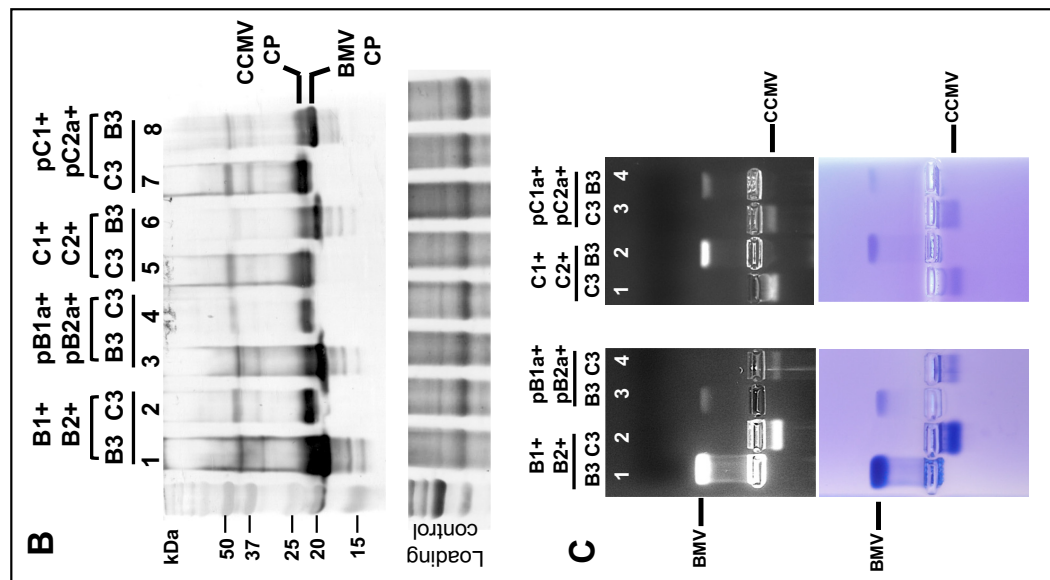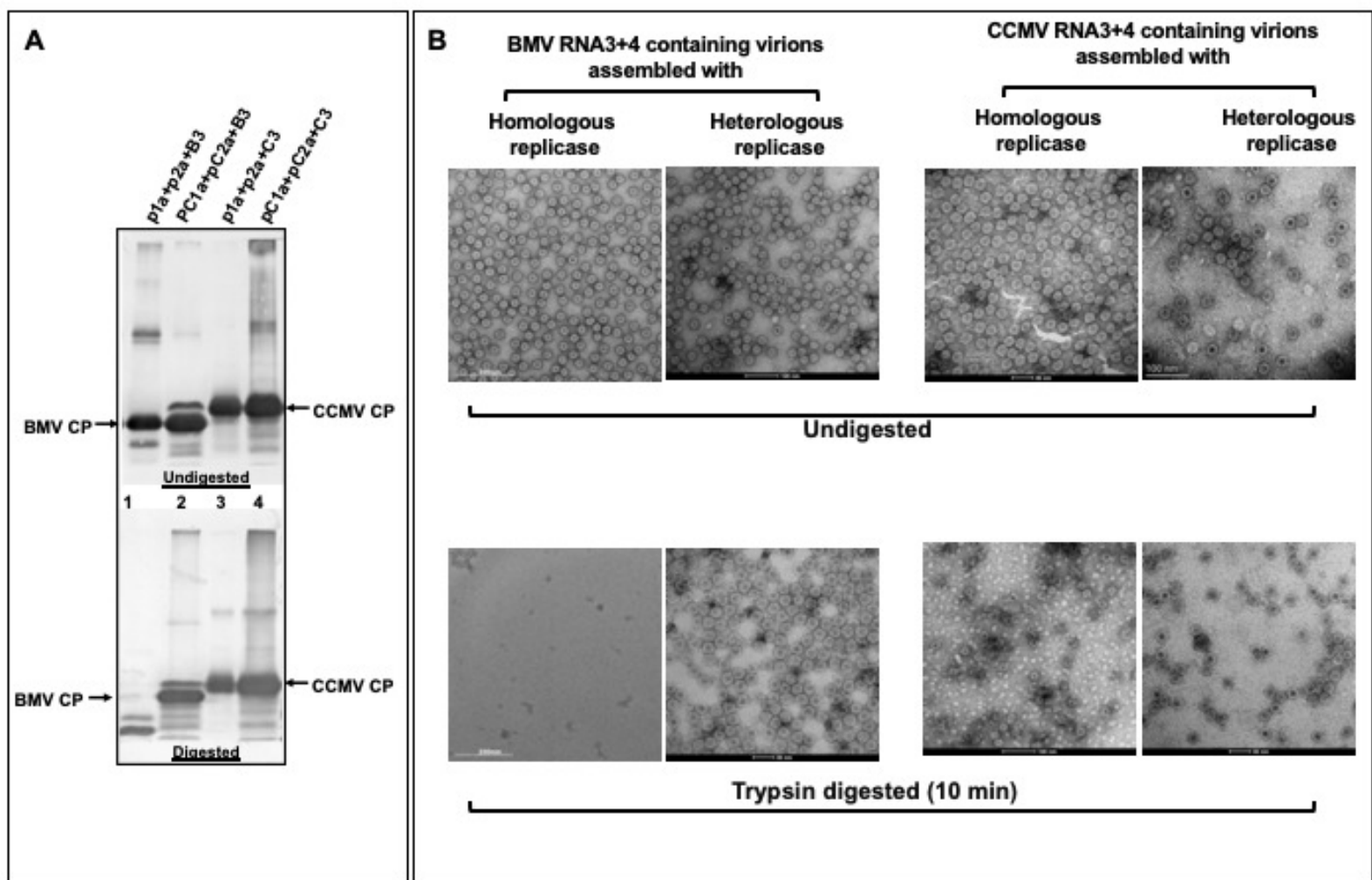
WT CCMV

A

B

C
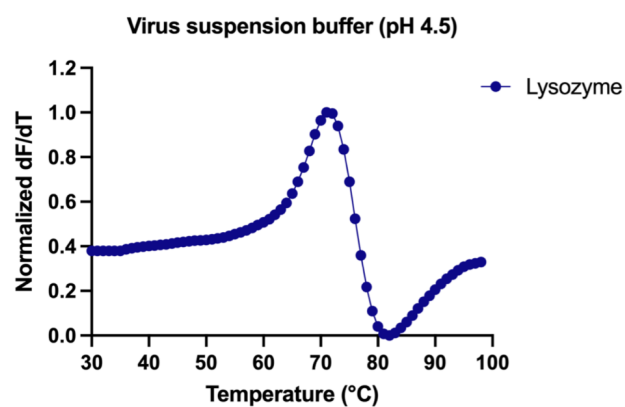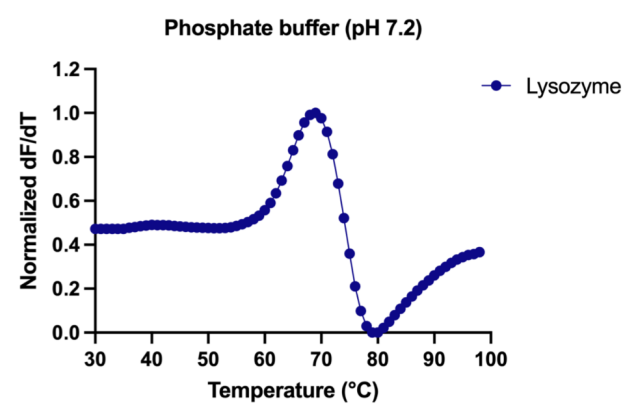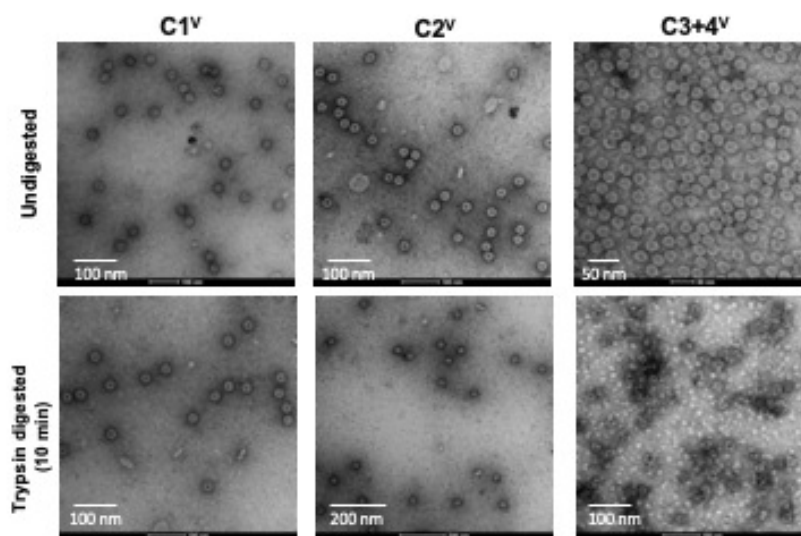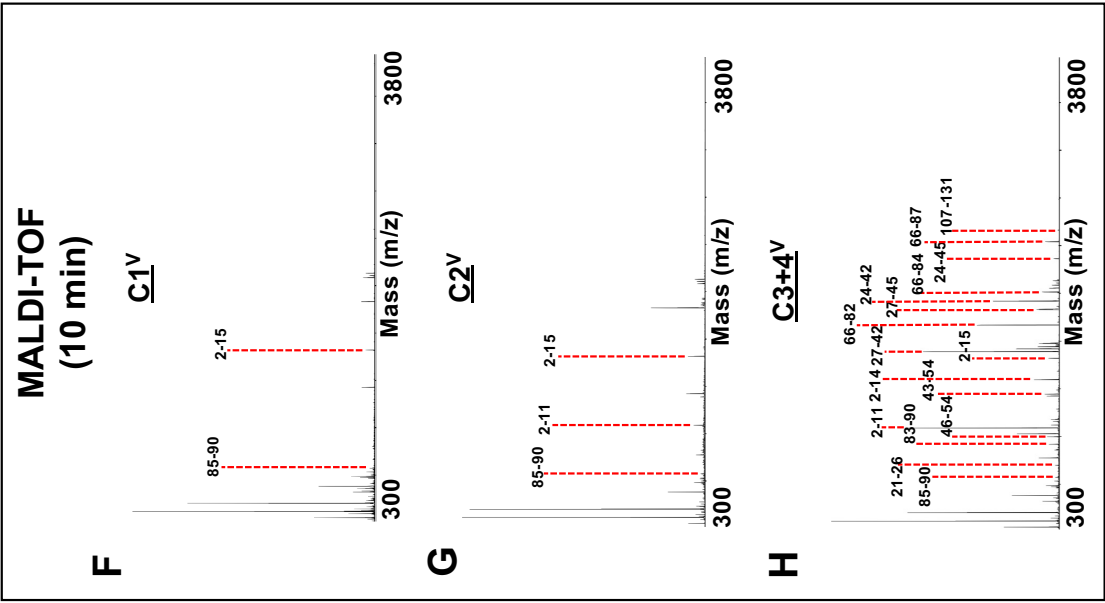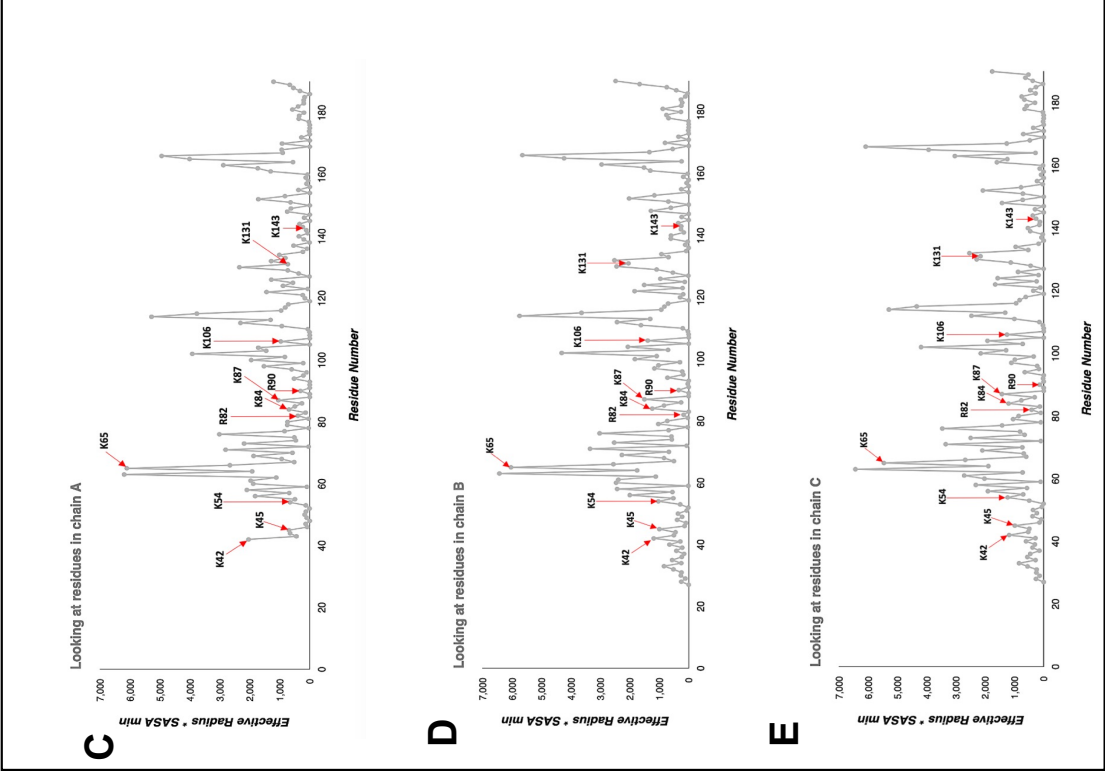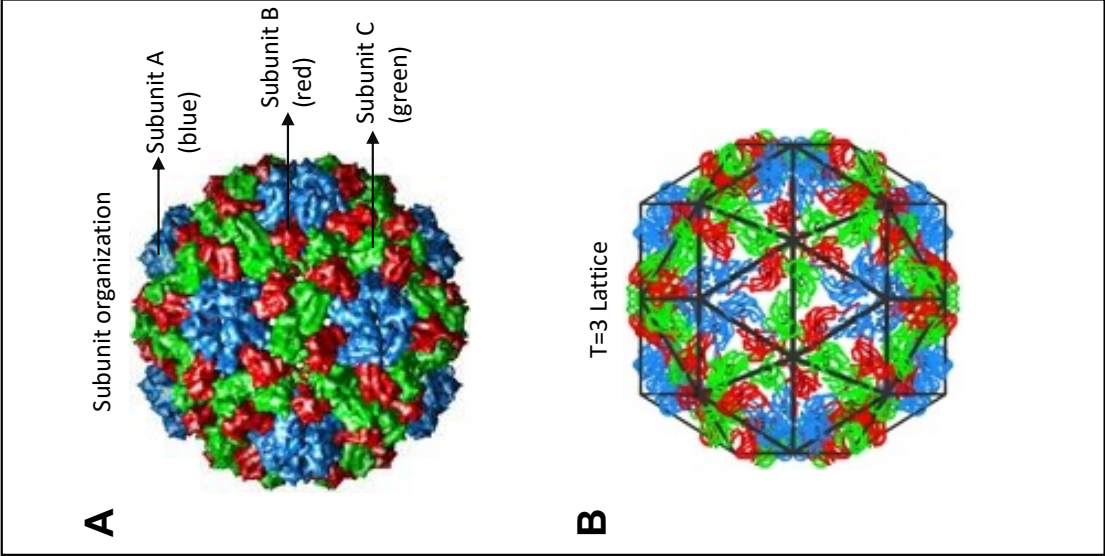
WT CCMV
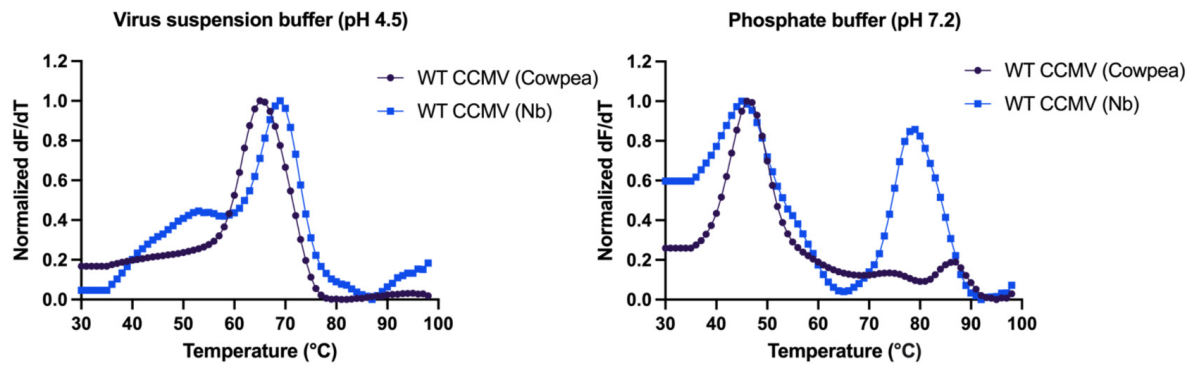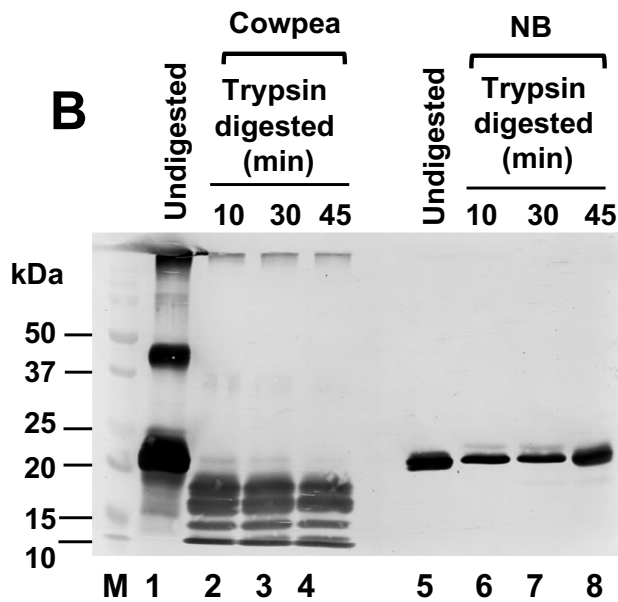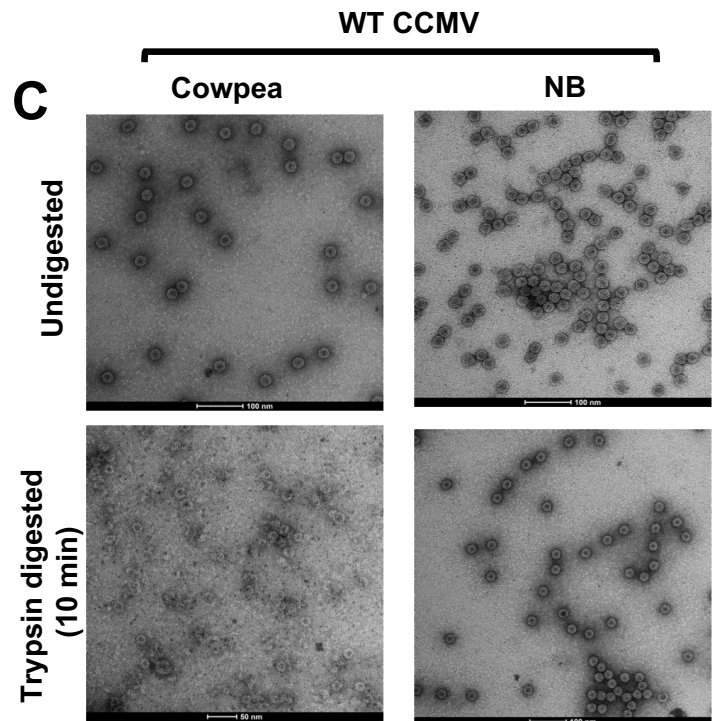
D
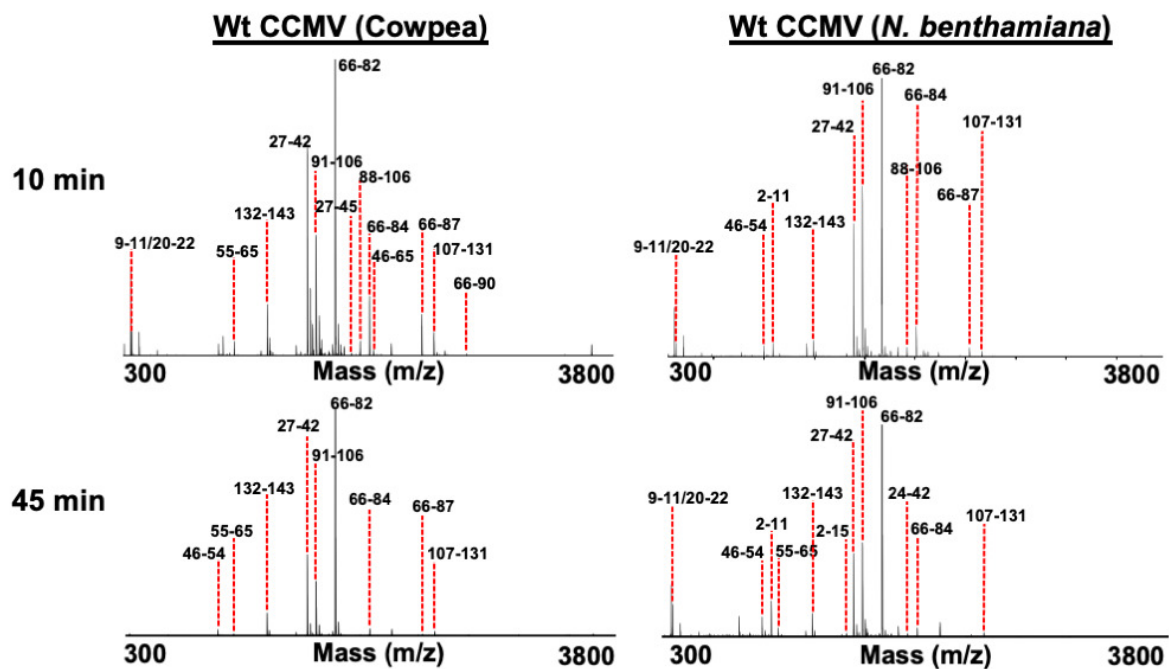
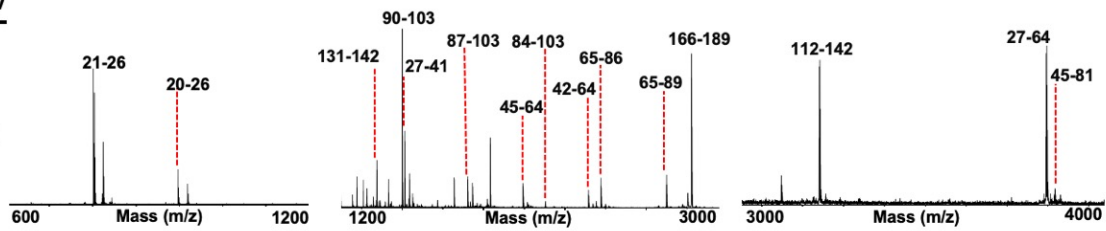**A. B3+4 virions with homologous BMV replicase**

B. B3+4 virions with heterologous CCMV replicase
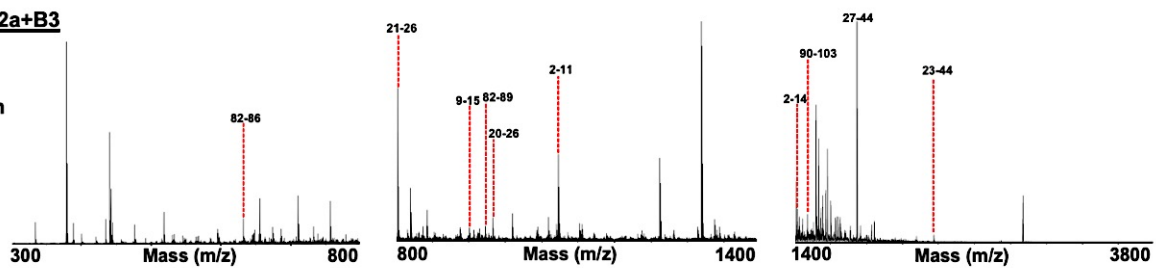
C. C3+4 virions with homologous CCMV replicase
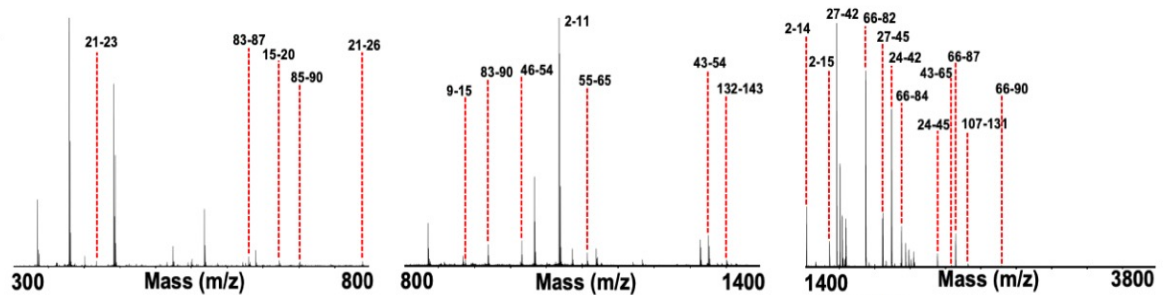
D. C3+4 virions with heterologous BMV replicase

Panel A

(i)

2-11, 2-14, 9-15, <12-20, 16-26, 20-26, 21-26, 23-44, 27-41, 27-64, 42-64, 45-64, 45-81, 65-86, 65-89, 82-86, 82-89, 84-103, 87-103, 90-103, 112-142, 131-142, 166-189

N — ARM — BMV CP — C

1 — 189

Total no. of trypsin cleavage sites= 24

(ii)

BMV CP subunits → *Assembled with homologous (i.e. BMV) replicase* (2a/1a) →

20-26, 21-26, 27-41, 27-64, 42-64, 45-64, 45-81, 65-86, 65-89, 84-103, 87-103, 90-103, 112-142, 131-142, 166-189

→ *Trypsin treatment* → **Unstable**

Total no. of trypsin cleavage sites= 15

(iii)

BMV CP subunits → *Assembled with heterologous (i.e. CCMV) replicase* (1a/2a) →

2-11, 2-14, 9-15, 20-26, 21-26, 23-44, 27-44, 82-86, 82-89, 90-103

→ *Trypsin treatment* → **Stable**

Total no. of trypsin cleavage sites= 10

Panel B

(i)

2-11, 2-14, 2-15, 9-11, 9-15, 15-20, 16-20, 16-23, 21-23, 21-26, 24-42, 24-45, 27-42, 27-45, 43-45, 43-54, 43-65, 46-54, 55-65, 66-82, 66-84, 66-87, 66-90, 83-87, 83-90, 85-90, 88-106, 91-106, 107-131, 132-143

N — ARM — CCMV CP —

1 — 190

Total no. of trypsin cleavage sites= 30

(ii)

CP subunits → *Assembled with homologous (i.e. CCMV) replicase* (2a/1a) →

2-11, 2-14, 2-15, 9-15, 15-20, 21-23, 21-26, 24-42, 24-45, 27-42, 27-45, 43-54, 43-65, 46-54, 55-65, 66-90, 66-97, 66-84, 66-82, 83-87, 83-90, 85-90, 107-131, 132-143

→ **Trypsin treatment** → **Stable**

Total no. of trypsin cleavage sites= 24

(iii)

CP subunits → *Assembled with heterologous (i.e. BMV) replicase* (2a/1a) →

2-11, 2-14, 2-15, 9-15, 16-20, 21-23, 27-42, 66-82, 91-106, 132-143

→ **Trypsin treatment** → **Stable**

Total no. of trypsin cleavage sites= 10