



**HAL**  
open science

## Nonconcomitant host-to-host transmission of multipartite virus genome segments may lead to complete genome reconstitution

Jérémy Di Mattia, Babil Torralba, Michel Yvon, Jean-Louis Zeddam, Stéphane Blanc, Yannis Michalakis

### ► To cite this version:

Jérémy Di Mattia, Babil Torralba, Michel Yvon, Jean-Louis Zeddam, Stéphane Blanc, et al.. Non-concomitant host-to-host transmission of multipartite virus genome segments may lead to complete genome reconstitution. *Proceedings of the National Academy of Sciences of the United States of America*, 2022, 119 (32), pp.e2201453119. 10.1073/pnas.2201453119 . hal-03755700

**HAL Id: hal-03755700**

**<https://hal.science/hal-03755700>**

Submitted on 22 Aug 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

1

2 **Main Manuscript for**

3 Non-concomitant host-to-host transmission of multipartite virus  
4 genome segments may lead to complete genome reconstitution.

5

6 Jérémy Di Mattia<sup>1</sup>, Babil Torralba<sup>1</sup>, Michel Yvon<sup>1</sup>, Jean-Louis Zeddami<sup>1</sup>, Stéphane Blanc<sup>1#</sup> and  
7 Yannis Michalakis<sup>2#\*</sup>

8 1: PHIM, Université Montpellier, INRAE, CIRAD, IRD, Institut Agro, Montpellier, F 34398,  
9 France

10 2: MIVEGEC, Université Montpellier, CNRS, IRD, Montpellier, F- 34394, France

11 # YM and SB equally contributed to this work

12 \* Yannis Michalakis

13 **Email:** yannis.michalakis@ird.fr

14 **Author Contributions:** All authors designed the experiments and wrote the manuscript; JDM  
15 and BT performed the experiments; YM analyzed the data.

16 **Competing Interest Statement:** The authors declare no competing interests.

17 **Classification:** Biological Sciences; Microbiology.

18 **Keywords:** multipartite virus, virus transmission, genome architecture, vector transmission,  
19 reassortment.

20

21 **This PDF file includes:**

22 Main Text  
23 Figures legends  
24

25 **Abstract**

26 Because multipartite viruses package their genome segments in different viral particles, they face  
27 a potentially huge cost if the entire genomic information, i.e. all genome segments, needs to be  
28 present concomitantly for the infection to function. Previous work with the octapartite faba bean  
29 necrotic stunt virus (FBNSV, Family *Nanoviridae*, Genus *Nanovirus*) showed that this issue can be  
30 resolved at the within-host level through a supra-cellular functioning: all viral segments do not need  
31 to be present within the same host cell, but may complement each other through inter-cellular  
32 trafficking of their products (protein or mRNA). Here, we report on whether FBNSV can as well  
33 decrease the genomic integrity cost during between-host transmission. Using viable infections  
34 lacking non-essential virus segments, we show that full genome infections can be reconstituted  
35 and function through separate acquisition and/or inoculation of complementary sets of genome  
36 segments in recipient hosts. This separate acquisition/inoculation can occur either through  
37 transmission of different segment sets by different individual aphid vectors, or by the sequential  
38 acquisition by the same aphid of complementary sets of segments from different hosts. The  
39 possibility of a separate between-host transmission of different genome segments thus offers a  
40 way to at least partially resolve the genomic maintenance problem faced by multipartite viruses.

41 **Significance Statement**

42 Multipartite viruses package each of their genome segments in different particles, facing a  
43 potentially huge cost if the entire genomic information needs to be present concomitantly. Previous  
44 work with the octapartite faba bean necrotic stunt virus (FBNSV) showed this issue can be resolved  
45 at the within-host level through a supra-cellular functioning: all viral segments do not need to be  
46 present within the same host cell, but may complement each other through inter-cellular trafficking  
47 of their products (protein or mRNA). Here we show that full genome infections can be reconstituted  
48 and function through separate acquisition and/or inoculation of complementary genome segment  
49 sets in recipient hosts, thus decreasing the genomic integrity cost during between-host  
50 transmission through a supra-host functioning.

51  
52

53 **Main Text**

54  
55

56 **Introduction**

57

58 About >25% of annotated viral species have their genomic information carried by more than one  
59 nucleic acid molecule (1). The most well-known are the “segmented viruses” which package all  
60 their genome segments in a single virion for transmission, such as the Influenza virus A. The  
61 “multipartite viruses”, despite being more numerous (~17% of all viral species) are much less  
62 studied, perhaps because their vast majority infects plants or fungi; these viruses package each  
63 segment in a distinct viral particle.

64

65 Multipartite viruses have important economical and plant health consequences, but at present  
66 remain an evolutionary puzzle: while none of the proposed advantages is specific to this genomic  
67 architecture (all are shared with segmented viruses but a surprising and idiosyncratic increased  
68 lifespan of viral particles observed in an artificial system (2)), they face a potentially huge cost if the  
69 entire genomic information, i.e. all genome segments, needs to be present concomitantly for the  
70 infection to function (1, 3). Indeed, if the different segments were transmitted randomly, an  
71 unrealistically large number of viral particles would need to enter each host cell (multiplicity of  
72 infection; MOI), particularly for multipartite viruses with more than four segments (4, 5).

73

74 Our work with the aphid-transmitted octapartite faba bean necrotic stunt virus (FBNSV, Family  
75 *Nanoviridae*, Genus *Nanovirus*) has added to the puzzle. The FBNSV genome is composed of

76 eight circular single stranded DNA segments, each of about 1000 bases and encoding a single  
77 protein (6, 7). Segment C encodes the cell cycle-linked protein (Clink), M encodes the movement  
78 protein (MP), N encodes the nuclear shuttle protein (NSP), S encodes the coat protein (CP), R  
79 encodes the master replication-associated protein (M-Rep), and U1, U2, and U4 encode proteins  
80 of unknown functions. We previously showed that (i) the eight genome segments do not occur at  
81 equal frequencies within host plants; the frequency distribution converges to a host plant species  
82 specific distribution, which we termed “genome formula” (8). The discovery of the genome formula,  
83 subsequently revealed in another nanovirus species (9) and in two unrelated multipartite viruses  
84 (10, 11), other than unveiling another biological ‘weirdness’ of these viruses begging for an  
85 explanation, further questions their existence: if some segments are rare the MOI needs to be even  
86 higher. (ii) We subsequently showed that within individual hosts all viral segments do not need to  
87 be present within the same host cell, but that they may complement each other through inter-  
88 cellular trafficking of their expression products (mRNA and/or protein); this distributed mode of  
89 functioning should greatly reduce the within-host putative genomic integrity cost (12). Nevertheless,  
90 the potential genomic integrity cost during host-to-host transmission still needs to be addressed.  
91 We directly showed that very few copies of each segment were aphid-transmitted from plant to  
92 plant, two to three orders of magnitude fewer than predicted from evolutionary models (5). This  
93 result, which empirically illustrates the between-host cost, was in agreement with the only other  
94 report on transmission bottlenecks in multipartite viruses, which found that when an aphid  
95 successfully inoculated the tripartite cucumber mosaic virus to a host plant, only one or two copies  
96 of each segment were transmitted (13).

97  
98 A possibility to circumvent the cost of host to host transmission, would be that the concomitant  
99 transmission of all genome segments is not necessary: the segments could be sequentially  
100 acquired by the same aphid from different host plants and then transmitted, or different aphids  
101 could acquire different segments from different plants and then transmit them to the same plant  
102 (14). In this paper, we partly tested this hypothesis. We took advantage of the fact that, although  
103 all segments were found in the ten known FBNSV field isolates (15–18), within host infection can  
104 be achieved in the lab if some of them are not present (19, 20). We inoculated different sets of  
105 plants with incomplete viral genomes, FBNSV<sup>C-</sup>, FBNSV<sup>N-</sup> or FBNSV<sup>U4-</sup>, each lacking the genome  
106 segment indicated in superscript. It has been previously shown that the absence of these segments  
107 does not affect within plant virus accumulation (20). We then tested whether the complete viral  
108 genome could be reconstituted on receiver plants, either following transmission by different aphids  
109 that fed on the different sets of plants, the parallel mode, or by the same aphid fed sequentially on  
110 the different plants, the sequential mode (Experiment 1). In the latter case, we also looked at how  
111 temporal spacing of the sequential acquisitions affected the probability of complete genome  
112 reconstitution (Experiment 2; see Figure 1 for a schematic of the two experiments; all experimental  
113 procedures are detailed in the Materials and Methods). Our results show that it is indeed possible  
114 to reconstitute the whole virus genome, in both cases, even though this is less likely when  
115 increasing the time between sequential acquisitions. We further show that under sequential  
116 acquisition the reconstitution occurs at the very early stage of the virus cycle within the aphid midgut  
117 cells. Our findings thus support the hypothesis that multipartite viruses may circumvent the genomic  
118 integrity maintenance cost during host-to-host transmission because concomitant transmission of  
119 all genome segments is not necessary. Their capacity to transmit genomic segments non-  
120 concomitantly suggests that these viruses have an immense potential to exchange genetic

121 information through reassortment. i.e. exchange of complete segments, since parental genotypes  
122 could exchange segments without ever occurring on the same host individual.

## 123 124 **Results**

### 125 126 **i. FBNSV full genome reconstitution is possible through both parallel and sequential** 127 **transmission by aphid vectors.**

128  
129 Figure 2 presents the proportions of infected plants and of those containing all eight segments, for  
130 all conditions and replicates without any temporal spacing between sequential acquisitions. Though  
131 with variable proportions, all conditions generated full FBNSV genome reconstitutions,  
132 demonstrating that such reconstitution is possible from infections with incomplete genomes,  
133 whether transmitted separately by different aphids (parallel transmission) or acquired sequentially  
134 by the same aphid (sequential transmission).

135  
136 In all replicates/conditions but two, some infections did not result in the reconstitution of the  
137 complete genome: the proportion of reconstitutions (red bars) was lower than that of infections  
138 (blue bars). Some segments are missing in these cases, and this is discussed in more length in a  
139 separate section.

140  
141 Sequential transmissions performed by single aphids led, over all conditions and replicates, to  
142 approximately 40% infection, compatible with what we previously found when transmitting the full  
143 genome with one aphid (5). Parallel transmissions performed by two aphids led to approximately  
144 60% infection. This transmission rate is very close to 64%, the proportion expected if each aphid  
145 had a 40% chance to transmit the infection and there was no interaction between them. Sequential  
146 transmission, however, led to roughly twice as many reconstitutions of the complete genome than  
147 parallel transmissions. Over all test plants, the 'grand mean' of those containing all eight FBNSV  
148 segments after parallel transmission is 13.6% while it is of the order of 25% after sequential  
149 transmission. This observation holds whether we calculate the mean over both experiments or only  
150 the "parallel vs sequential" experiment, over both pairs of segments or only over C/U4 (which are  
151 the only segments involved in parallel transmissions). It is worth noting, however, that the  
152 differences in reconstitution rate between parallel and sequential transmission modes are due only  
153 to the U4-||C- treatment (with the data of only the first experiment  $z=2.011$ ,  $p=0.044$ ; with the data  
154 of both experiments in Fig. 2  $z=2.240$ ,  $p=0.025$ ). The reconstitution rates of the C-||U4- reverse  
155 acquisition order does not differ statistically from those of the parallel transmission treatment (with  
156 the data of only the first experiment  $z=0.046$ ,  $p=0.963$ ; with the data of both experiments in Fig. 2  
157  $z=0.27$ ,  $p=0.821$ ).

158  
159 We further investigated whether the missing segments and their order of acquisition in sequential  
160 transmission affected the rates of infection and reconstitution. As already suggested by the above  
161 comparison looking at the situations involving segments C and U4, despite variation among  
162 replicates, there were more infections and reconstitutions when segment C was acquired first:  
163 across both experiments and replicates, U4-||C- led to ~54% infections and ~32% reconstitutions  
164 vs. ~31% infections and ~15% reconstitutions when C was acquired second (C-||U4-). These  
165 differences were statistically significant ( $z=2.496$ ,  $p=0.013$ ; the 'experiment' effect was not  
166 statistically significant  $z=0.061$ ,  $p=0.951$ ) and are further discussed later.

167  
168 For N and U4 combinations, it is highly remarkable that transmission was efficient even when  
169 segment N was not acquired first (i.e. the N-||U4- conditions). Indeed, it has been repeatedly shown  
170 that no transmission occurs from plants lacking segment N (14, 15), which encodes for the helper  
171 factor of FBNSV (14). We comment on this surprising result more extensively in the Discussion  
172 section. For the moment we note that, even though more variable between replicates,  
173 transmissions involving segments N and U4 led to similar proportions of infection and reconstitution  
174 as transmissions involving segments C and U4. However, in contrast to segment C and U4, the

175 reconstitution rates resulting from the two N and U4 acquisition orders did not differ significantly  
176 ( $z=0.181$ ,  $p=0.856$ ; 'experiment'  $z=-0.908$ ,  $p=0.364$ ; 'experiment'-by-'order of acquisition'  
177 interaction  $z=0.633$ ,  $p=0.526$ ).

178  
179 **ii. FBNSV infection and reconstitution when sequential acquisition by aphids is spaced**  
180 **in time**

181  
182 Figure 3 presents the proportion of infected plants (blue) and of plants with the full genome  
183 reconstituted (red) when segments were acquired sequentially but with variable spacing in time  
184 of 0, 1, 2 or 3 days. The figure clearly shows that increasing spacing in time rapidly leads to a  
185 decrease both in infection and reconstitution rates (infection:  $z=-3.310$ ,  $p<0.001$ ; reconstitution:  $z=-$   
186  $2.567$ ,  $p=0.010$ ). Indeed, with no spacing (0 d), across all segment combinations and replicates, 65  
187 plants were infected and 38 had the full genome reconstituted out of a total of 170 receiver plants,  
188 while after 3 d spacing only 13 plants were infected and only one had a reconstituted genome over  
189 a total of 174. Most infections and reconstitutions occurred with no or just one day spacing, while  
190 very little of either occurred with more than one day.

191  
192 None of the order of acquisition comparisons revealed a statistically significant effect except for the  
193 N-||U4- condition which resulted in less infections ( $z=-2.148$ ,  $p=0.032$ ).

194  
195 **iii. Incomplete genome reconstitution and loss of segments**

196  
197 As mentioned earlier, many infections did not result in the reconstitution of the complete FBNSV  
198 genome, implying that at least one segment was missing. Here, we summarize the broad patterns,  
199 first on the replicates without time spacing and then on the replicates of time spacing under  
200 sequential transmission, and relegate the detailed account to SI tables S1 and S2.

201  
202 Among the 385 receiver plants used in all segment combinations and transmission mode (without  
203 time spacing), 158 were infected. Only three of those did not carry segment N indicating that this  
204 segment is rarely involved in incomplete or failed reconstitutions. Segment C was absent from  
205 14.5% (23/158) infected plants and there was no clear pattern in the distribution of these cases,  
206 either between transmission modes or across segment combinations involved. Segment U4 was  
207 absent from 33% (52/158) infected plants. It was particularly absent from recipient plants of the  
208 parallel transmission mode (63%), but much less so in all sequential transmission mode treatments  
209 (27%), with no strong pattern across segment combinations involved.

210  
211 Time spacing had no effect on segment N which was present in all infected plants except for the  
212 plants mentioned above plus two more plants. Segment C was present in ~80% of infected plants  
213 for all time-spacing intervals except for 3 d where it was present in only 6/13 infected plants.  
214 Segment U4's presence gradually declined with time-spacing interval, being present in 75%  
215 infected plants at 0 d spacing, and down to 38% at 3 d. No clear pattern could be observed across  
216 segment combinations involved (table S2).

217  
218 **iv. Reconstitution may occur already in aphids upon sequential acquisition**

219  
220 Using segment-specific Fluorescence In Situ Hybridization (FISH) by specifically labeling the pairs  
221 of sequentially acquired segments (segment C then U4 or N then U4), we investigated whether the  
222 FBNSV genome could reconstitute in aphid vectors, when subsets of segments were acquired  
223 sequentially. Aphids were placed on plants during 7 days for each acquisition step, with one day  
224 on healthy plants between the two acquisition steps and one day of feeding on water through a

225 Parafilm membrane after the second acquisition to eliminate the virus present in the lumen of the  
226 aphid midgut.

227  
228 For segments C and U4, when U4 was acquired first (i.e. C-||U4- treatment), a few intracellular  
229 fluorescent foci showed co-localization of the two segments (Figure 4A white arrows). However,  
230 the majority contained only one of the two, rare foci containing only U4 (green) and numerous  
231 others containing only C (red). In contrast, when C was acquired first (U4-||C-), a large proportion  
232 of the foci contained both segments (Figure 4B). Even more remarkable, for segments N and U4  
233 we observed a near perfect co-localization in all foci (Figures 4C and D) for both acquisition orders  
234 (U4-||N- or N-||U4-). These observations were very consistent across biological replicates: 16, 10,  
235 6 and 14 individual aphids were examined for treatments C-||U4-, U4- ||C-, U4-||N- and N-||U4-  
236 respectively. At least ten cells of the anterior midgut were zoomed for each individual aphid. For  
237 each treatment, all zoomed cells revealed the same pattern, illustrated in Figure 4. All controls for  
238 these experiments, particularly those confirming the segment-specificity of the probes are shown  
239 in figure S1 (see the “Without C” and “without U4” panels).

240  
241 Altogether, our results demonstrate that full genome reconstitution can occur at very early stages  
242 of the virus cycle within the aphid vectors, soon after entry of the segments into midgut cells.

243  
244 **v. The reconstituted viral genome can be transmitted as an integral FBNSV genome**

245  
246 We finally verified that the FBNSV could be aphid-transmitted from plants containing a reconstituted  
247 genome in a way similar to that observed from plants initially agro- or aphid inoculated with the  
248 complete genome, as detailed in the Materials and Methods. Over the four transmission assays  
249 (one donor plant with a reconstituted genome from each of the sequential transmission treatments  
250 C-||U4-, U4-||C-, N-||U4- and U4-||N-), we obtained 36.36% (28/77) successful transmissions. This  
251 transmission rate is close to that we typically observe for transmissions by a single aphid under our  
252 experimental conditions, from plants initially infected with full FBNSV genome. There was variability  
253 among treatments, at least partially reflecting the relatively small sample sizes (U4-||C-: 45.45%  
254 (10/22); C-||U4-: 31.25% (5/16); U4-||N-: 50% (10/20); N-||U4-: 15.79% (3/19)).

255  
256 In the 28 infected plants, we observed the absence of one or two segments in only 3 plants:  
257 segment U4 was absent from one plant of the U4-||C- treatment, segments C and N were both  
258 absent from one plant of the C-||U4- treatment and segments C and U4 were both absent from  
259 another plant of C-||U4- treatment. Finally one plant of the U4-||C- tested negative for segments N  
260 and U4 and had one qPCR replicate slightly above ( $C_t = 30.85$ ) and one slightly below ( $C_t = 30.28$ )  
261 the threshold value of segment C ( $C_t = 30.43$ ). Repeating the qPCRs for this individual plant yielded  
262 almost identical values. We decided to consider this plant as not infected as indicated by most of  
263 the evidence.

264  
265 These results thus confirm that the reconstituted FBNSV can successfully be transmitted by aphids  
266 to uninfected plants as the integral viral genome.

## 267 268 269 **Discussion**

270  
271 Our results clearly show that the reconstitution of the complete FBNSV genome through aphid  
272 transmission from distinct plants infected with incomplete genomes is possible. This result  
273 demonstrates that all FBNSV segments do not need to be acquired and/or inoculated  
274 concomitantly, and thus has important implications for our understanding of how multipartite viruses  
275 may be coping with the so far assumed cost of a multipartite way of life related to the maintenance  
276 of their genomic integrity during host-to-host transmission (1, 3–5, 14, 21).

277

278 The non-concomitant transmission of genomic segments could potentially occur in all vector-borne  
279 multipartite viruses, even those not following the circulative transmission mode. There is no reason  
280 why the parallel mode would be restricted to such viruses. The relevance of the sequential mode  
281 would depend on the opportunity of viral particles from different acquisitions to co-occur in the  
282 vector. This should not be a problem for semi-persistent viruses which may be retained in their  
283 vectors from hours to days (e.g. the bipartite criniviruses transmitted by whiteflies (22) to  
284 weeks/months/years (e.g. the bipartite nepoviruses and tobnaviruses transmitted by nematodes  
285 (23). Even non-persistent viruses could potentially use the sequential mode because they may  
286 cause alterations of their vectors' behavior that make the vectors visit more plants more rapidly  
287 (e.g. the tripartite CMV (24).

288

289 We found that genome reconstitution could occur when incomplete infections are transmitted either  
290 by distinct aphids, a mode we termed parallel transmission, or by the same aphid sequentially  
291 acquiring viral segment sets from different plants to transmit them together to a receiver plant.  
292 These modes represent the two extremes of a continuum; reconstitutions could potentially, and  
293 probably most likely, occur in nature through any mixture of these modes.

294

295 When transmission is parallel, reconstitution can only occur in the receiver plant. Our results show  
296 that the overall infection levels we observed, ~60%, are compatible with the independent  
297 transmission of the two incomplete infections by the two aphids that potentially carry them.  
298 However, only ~22% of these infected plants ended up with a reconstituted complete genome, the  
299 remaining plants lacking either segment C or, for unknown reasons, more frequently U4. These  
300 segments may be missing either because they were not transmitted, or because they were lost in  
301 the receiver plant after transmission. The former could occur because the aphids which were  
302 supposed to transmit them did not acquire, or acquired but did not inoculate them to the receiver  
303 plant in sufficient amounts. Consistent with this is our previous demonstration that very few viral  
304 particles are transmitted from single aphids (5), likely resulting in frequent segment loss sometimes  
305 resulting in infection failure. Unfortunately, this previous study quantifying the bottleneck that aphid  
306 transmission imposes to FBNSV populations did not specifically monitor the transmission of  
307 segments C and U4, and thus we have no data allowing direct comparison. If 'missing segments'  
308 were lost after transmission, we see no obvious reason explaining why segment U4 should be lost  
309 more often than segment C, as it is more frequent than segment C both in plants (8) and aphids  
310 (25). Our results on the transmission from plants with the reconstituted genomes indicate that  
311 segment loss is much rarer when donor plants have all eight segments, suggesting that  
312 transmission from plants with incomplete infections may be particular in this respect. This matter  
313 thus deserves further investigation. Incomplete infections are rarely reported on field isolates of  
314 nanoviruses, which could be due to several compatible explanations: a reporting bias, a lower  
315 performance relative to full infections or complementation of initially incomplete infections through  
316 the processes investigated here.

317

318 Sequential transmission without time-spacing led to infection levels in agreement with previous  
319 single-aphid transmission results (5). Reconstitution of the complete genome was much more  
320 frequent under this transmission mode: ~50% of infected plants carried all genome segments, as  
321 opposed to only 22% through parallel transmission. It is remarkable that the segments acquired  
322 sequentially co-localized within intracellular granules (fluorescent foci) in aphid gut epithelial cells,  
323 indicating that under this transmission mode reconstitution can occur at very early stages of the  
324 virus cycle within the body of its vector. This observation suggests similar accumulation sites for  
325 the eight FBNSV segments in aphid midgut cells, whether acquired concomitantly (26) or  
326 sequentially (this study), which may explain the higher reconstitution rate, when compared to  
327 parallel transmission.

328

329 We found an unexpected effect due to the order of acquisition: more infections and reconstitutions  
330 occurred when segment C was acquired first ('U4-||C-' treatment) than second ('C-||U4' treatment).  
331 The fact that we only rarely manage to obtain infections lacking segment C through agro-inoculation



332 may suggest that, at least under our conditions, this segment plays an important role which was  
333 not revealed in other experimental conditions (20). Alternatively, we cannot exclude that segment  
334 C plays a yet unanticipated role in FBNSV transmission in the aphids, resulting into higher  
335 transmission if it is acquired early.

336

337 A much more remarkable result, however, concerns segment N. As previously stated, it is  
338 established that the presence of this segment is mandatory for aphid transmission (20, 27). It is  
339 thus considered as the segment producing the helper component required for FBNSV transmission  
340 (28). So far, all described helper components in the vector-transmission of plant viruses must be  
341 acquired together with or before the viral particles (29–32). Our sequential experiment results,  
342 however, clearly demonstrate that this is not the case for the N segment of FBNSV: it is possible  
343 to have transmission, successful infection and even full genome reconstitution even if segment N  
344 (and/or its expression products) is acquired after the other segments (i.e. our 'N-||U4-' treatment).  
345 This suggests that the segments acquired by the aphid from plants that do not contain the segment  
346 N can 'survive' or wait in the aphid's gut until segment N (and/or its expression products) are  
347 acquired during the second acquisition step. The single full genome reconstitution (out of a total of  
348 13 infected plants) we observed with a 3d-spacing shows that this complementation can occur with  
349 at least this delay between the two acquisition steps. We have previously shown that when the  
350 virus is acquired from plants lacking segment N no viral segment is visible in aphid midgut cells,  
351 while when N is present in the infected plant they all penetrate and accumulate together into  
352 cytoplasmic granules (27). Our confocal microscopy results show that the same process occurs  
353 during the sequential acquisition, whether N is acquired prior to or after the other segments. The  
354 specific mechanisms through which the helper of FBNSV acts need further studies. We can note,  
355 however, more to the point of this study, that by being able to potentiate transmission of viral  
356 segments previously or subsequently acquired by aphids, this helper allows for a much larger  
357 spectrum of possible reconstitutions of the virus' full genome. Consequently, genome reconstitution  
358 through sequential acquisition and transmission may help FBNSV, and potentially other  
359 nanoviruses, alleviate the cost of maintenance of genome integrity upon host-to-host transmission.

360

361 Our time-spacing results were very surprising. We observed that infection and reconstitution  
362 declined as time-spacing increased. FBNSV, as all nanoviruses, is supposed to be transmitted  
363 according to the circulative non-propagative mode (33), and thus travel through the aphid's body,  
364 gut and salivary gland cells, without replicating. Depletion of the first acquired infection (drastic  
365 reduction of transmission rate) during the short time intervals we used is thus surprising (34). But  
366 what is even more surprising is that the second-acquired infection should lead to successful  
367 infections on its own, even if the first infection was depleted, at a rate that in principle should not  
368 depend on the time since the first acquisition. This is clearly not what happened in our experiments.  
369 One potential explanation could be that the ability of aphids to successfully transmit viral infections  
370 decreases with time. As already noted above, this would be unlikely within the short time intervals  
371 we used (34). An alternative explanation could be that the first acquisition somehow triggers a  
372 mechanism which inhibits transmission after the second acquisition, and the strength of this  
373 inhibitory mechanism would increase with spacing-time between the two acquisitions. Because it  
374 is beyond the scope of this work, we leave this very interesting phenomenon to the future  
375 investigation it deserves. We note for the moment that this unanticipated negative effect of time-  
376 spacing between sequential acquisitions may limit the potential of reconstitutions to help  
377 multipartite viruses overcome the cost linked to genomic integrity upon host-to-host transmission.

378

379 It is currently impossible to quantitatively evaluate the potential of complete genome reconstitution  
380 through non-concomitant transmission of different segments to alleviate the host-to-host  
381 transmission cost of multipartitism. Such evaluation would require the precise knowledge of  
382 ecological and behavioral variables, such as the prevalence and density of infected plants, the  
383 density of vectors, how often vectors move from plant to plant, what governs their individual host  
384 plant choice, all of which is currently unknown. Moreover, in the introduction, we stated that our  
385 study "partly" addresses this issue. We used this term because we acknowledged that here we

386 demonstrate that reconstitution is possible between incomplete infections which are viable (even  
387 though they do not necessarily perform as well as complete genome infections). The potential of  
388 genome reconstitution to alleviate the cost of maintenance of genomic integrity would be much  
389 larger if it could be shown that such reconstitution occurs also from uptake within aphids or release  
390 within plants of segments sets which are not viable on their own. We relegate this to future  
391 investigation. This study nevertheless demonstrates that the concomitant transmission of the  
392 different genome components is not mandatory. Thus after showing that multipartite viruses may  
393 solve the within-host cost of genomic integrity maintenance through a supra-cellular, distributed,  
394 functioning (12), the present study shows that they may solve the between-host cost of genomic  
395 integrity maintenance through a supra-individual host, distributed, transmission. Further, this  
396 functioning suggests that these viruses have an immense potential for reassortment; compared to  
397 e.g. segmented viruses which in order to reassort need to co-infect the same host cell, since  
398 parental genotypes could exchange segments without ever having to 'meet' each other, a feature  
399 that may greatly influence their evolution.  
400

401  
402

## 402 **Materials and Methods**

403  
404

### 404 **i. Virus isolate, clones and plant agroinoculation**

405  
406

405 The first FBNSV isolate, discovered on faba beans (*Vicia faba*) in Ethiopia (15) was characterized  
406 and cloned by Grigoras and colleagues (35). Each genome segment was inserted as a head-to-tail  
407 dimer into the binary plasmid pBin19. Eight plasmids, each containing a dimer of a segment,  
408 together constitute the FBNSV infectious clone. The impact on plant infection and plant-to-plant  
409 transmission of the absence of any of the segments was investigated (20). The authors showed  
410 that the presence of segments R, S and M was mandatory for infection but it was possible to infect  
411 plants when one of segments C, N, U1, U2 or U4 was missing. They further found that the absence  
412 of segments C or U4 did not affect virus accumulation and transmission while the absence of U1  
413 or U2 decreased symptom severity and virus titer. They also established that the absence of the N  
414 segment impedes plant-to-plant aphid-transmission, even though it does not seem to affect within-  
415 plant viral growth.  
416

417  
418

417 In this study we inoculated ten-day-old faba bean plantlets (*Vicia faba* cv. Sevilla; Vilmorin) with  
418 FBNSV<sup>complete</sup>, FBNSV<sup>C-</sup>, FBNSV<sup>N-</sup> or FBNSV<sup>U4-</sup> agrobacteria solutions (strain COR308), as earlier  
419 described (35). While FNSV<sup>complete</sup> corresponds to a mixture at equal ratio of eight agrobacterium  
420 cultures, each containing one of the eight plasmids described above, the indicated culture and thus  
421 the indicated segment was omitted in FBNSV<sup>C-</sup>, FBNSV<sup>N-</sup> or FBNSV<sup>U4-</sup>. Plants were maintained as  
422 previously described in (27). Plant infection was controlled by total DNA extraction and qPCR (see  
423 below) 21 days after agro-inoculation. Even though we were able to obtain relatively many infected  
424 plants through agro-inoculation of FBNSV<sup>N-</sup> and FBNSV<sup>U4-</sup>, respectively 51.06% (=96/188) and  
425 52.81% (=94/178), we could only obtain 13.08% (=56/428) infected plants with FBNSV<sup>C-</sup>.  
426

427  
428

### 428 **ii. Aphid transmission**

429  
430

429 For all experiments we used aphid colonies of *Acyrtosiphon pisum* (clone 210) maintained in a  
430 controlled chamber as previously described in (27). Transmission tests were made under similar  
431 conditions using faba beans as host plants.  
432

433  
434

433 We tested whether the complete virus genome could be reconstituted if: (i) different aphids fed on  
434 different plants carrying incomplete infections missing different segments came together on the  
435 same recipient plant, a mode we hereafter call 'parallel transmission'; and if the same aphid  
436 sequentially fed on different plants carrying incomplete infections missing different segments was  
437 then transferred on one recipient plant, a mode we hereafter call 'sequential transmission'. We use  
438 these terms for ease of language although the parallel mode implies separate acquisition as well  
439 as separate inoculation of the distinct sets of segments, while the sequential mode implies a

440 sequential acquisition of the distinct sets of segments and may allow their concomitant inoculation.  
441 Subsequently (ii), we asked whether sequential transmission could successfully reconstitute the  
442 complete viral genome if the sequential feedings of the aphid were separated in time (from 0 up to  
443 3 days). Finally (iii), we tested that the reconstituted genomes were successfully transmitted as the  
444 genuine parental genotype. Figure 1 provides a schematic representation of the experimental  
445 design.

446

447 Parallel vs. sequential transmission

448

449 In a first experiment we tested whether the complete genome could be reconstituted through  
450 parallel or sequential transmission and at which rate. For the parallel transmission test (Figure 1,  
451 blue arrows), two FBNSV<sup>C-</sup> and two FBNSV<sup>U4-</sup> plants, one of each per replicate, were used as  
452 donors and 40 aphids were placed on each plant. Aphids fed on plants during an acquisition access  
453 period (AAP) of 3 days to acquire the respective set of segments. Then, we took one aphid from  
454 each donor plant and placed them on two different leaflets of the same recipient plant in a clip-cage  
455 (cage allowing the maintenance the aphid on one leaflet) during an inoculation access period (IAP)  
456 of 3 days.

457

458 For sequential transmission, we used one FBNSV<sup>C-</sup> and two FBNSV<sup>U4-</sup> donor plants and further  
459 tested whether the order of acquisition of the different incomplete infections affected the probability  
460 of complete genome reconstitution. Thus, for each condition 40 aphids were placed on each donor  
461 plant (either FBNSV<sup>C-</sup> or FBNSV<sup>U4-</sup>) for the first acquisition step. Then, we directly transferred  
462 aphids on the plants for the second acquisition step with the complementary set of segments. Each  
463 AAP step lasted 3 days. Finally, aphids were individually transferred into a clip-cage on a leaflet of  
464 a receiver plant (one aphid/receiver) during an IAP of 3 days.

465

466 Transmission does not occur in the absence of segment N (20, 27), which is thus considered to  
467 encode for a transmission helper component (28, 36). For this reason, we used only incomplete  
468 infections lacking either segments C or U4 for the parallel transmission mode. For the sequential  
469 transmission mode, we used the following combinations: (1) FBNSV<sup>C-</sup> then FBNSV<sup>U4-</sup> (C-||U4-), (2)  
470 FBNSV<sup>U4-</sup> then FBNSV<sup>C-</sup> (U4-||C-), (3) FBNSV<sup>N-</sup> then FBNSV<sup>U4-</sup> (N-||U4-) and (4) FBNSV<sup>U4-</sup> then  
471 FBNSV<sup>N-</sup> (U4-||N-). The third combination allowed to test whether successful transmission of  
472 segments can occur even if the N segment, which is necessary for aphid transmission, cannot be  
473 acquired during the first but during the second acquisition.

474

475 The number of receiver plants per transmission mode and replicates are given in Table 1. All  
476 replicates for both transmission modes were conducted at the same time.

477

478 Sequential transmission spaced in time

479

480 We subsequently tested whether the time separating the two sequential acquisitions had an effect  
481 on the reconstitution of the complete viral genome.

482

483 For this type of experiments (Figure 1, purple arrows), 200 to 250 aphids were placed on a first  
484 donor plant infected with one set of segments. Then, 40 aphids were transferred on a second donor  
485 plant infected with the complementary set of segments. When the two sequential acquisitions were  
486 not spaced in time (0 day) the transfer occurred immediately after the end of the first AAP period.  
487 The remaining aphids were placed onto a healthy plant. Every 24 h, up to a maximum spacing of  
488 3 days, 40 aphids were taken from the healthy plant and placed on a second donor plant infected  
489 with the complementary set of segments. Each AAP lasted 3 days. Finally, at the end of the second  
490 AAP, aphids were individually placed in a clip-cage on a leaflet of a receiver plantlet during an IAP  
491 of 3 days.

492

493 Two replicates were carried out for each combination and spacing time (see Table 1). However,  
494 because the number of receiver plants involved was very large, we first conducted one replicate  
495 with all segment combinations and spacing times, and two weeks later, the second replicate.  
496 All IAP were stopped with a spray of insecticide (Pirimor – Certis) except where otherwise  
497 mentioned. For all experiments, 21 days after the end of the IAP, total DNA of symptomatic plants  
498 was extracted and qPCR was performed to test for the presence of segments C, N and U4 (see  
499 qPCR details below).

500  
501 Transmission of the reconstituted complete FBNSV genome

502  
503 To verify if the reconstituted complete FBNSV infections can be transmitted to healthy plants, just  
504 as the genuine parental genotype, we used the infected faba beans from the sequential  
505 transmission experiments with 1 day of time spacing as source plants. In this case, at the end of  
506 the IAP of the second replicate, plants were not treated with the insecticide solution. Instead, all  
507 aphids were individually removed from the plants and a spray of Marseille soap in water  
508 (approximately 1 weight/volume concentration) was applied onto the plants to eliminate potentially  
509 remaining aphids. We allowed for 21 days of disease progression during which plants were isolated  
510 to avoid any aphid contamination. After this period, one plant from each of the C-||U4-, U4-||C-, N-  
511 ||U4- and U4-||N- sequential transmission experiments with the reconstituted complete FBNSV  
512 genome (checked by qPCR) was used as donor for the transmission of the reconstituted virus.  
513 Forty aphids were placed onto one donor plant of each combination. After an AAP of 3 days, one  
514 aphid was placed onto each of 20 to 24 receiver plantlets for an IAP of 3 days. Finally, 21 days  
515 after the end of the IAP, total DNA of symptomatic receiver plants was extracted and the segments  
516 C, N and U4 were quantified by qPCR.

517  
518 **iii. Viral DNA extraction and quantitative PCR (qPCR) detection**

519  
520 Total DNA extraction from plants was performed as described in (37). FBNSV infection causes faba  
521 bean leaf rolling and plant stunting in the early stage of infection and chlorosis and necrosis in the  
522 late stage. We extracted DNA from all plants showing some signs of leaf rolling and stunting and  
523 quantified segments C, N or U4 in all these plants. On the two upper leaf-level of symptomatic faba  
524 beans, three circular area of 0.6 cm diameter were squashed onto a Whatman paper disc. Then,  
525 we deposited each disc onto the filter of a 200  $\mu$ L micropipette tip and added 100  $\mu$ L of modified  
526 Edwards buffer (200 mM Tris-HCl pH 7.5, 25 mM EDTA, 250 mM NaCl, 0.5% SDS, 1% PVP40,  
527 0.2% ascorbic acid in water). Samples were centrifuged, at 5000 g for 15 sec, into a PCR plate  
528 placed underneath. Finally, we precipitated the DNA with 50% isopropanol (final concentration) and  
529 washed the sample once with 70% EtOH before resuspending it in 50  $\mu$ L of distilled water.

530  
531 For the qPCR quantification of the C, N and U4 segments, each sample was diluted 10 times in  
532 distilled water. We used the LightCycler 480 thermocycler (Roche, Indianapolis, Ind, USA) using 2  
533  $\mu$ L of total DNA one-tenth diluted extract. We used the LightCycler FastStart DNA Master Plus  
534 SYBR Green I kit (Roche) according to the manufacturer's instruction. For each sample, we added  
535 5  $\mu$ L of the 2X qPCR Mastermix, 0.3 or 0.5  $\mu$ M primers (0.3  $\mu$ M final for segment C and 0.5  $\mu$ M for  
536 segments N and U4 – specific primers for FBNSV segments were described in (6)), complemented  
537 at 8  $\mu$ L with H<sub>2</sub>O. Two  $\mu$ L of DNA sample were added to a final mix of 10  $\mu$ L. All samples were  
538 analyzed with two technical replicates. Forty qPCR cycles of 95°C for 10 s, 60°C for 10 s and 72°C  
539 for 10 s were applied to the samples. Post-PCR data analyses were carried as described elsewhere  
540 (38).

541  
542 Because the goal of this study is to investigate whether complete infections can be reconstituted  
543 from incomplete infections, we need to define values of Ct, i. e. the number of cycles needed to  
544 reach the fluorescence threshold, beyond which a segment can be considered as absent. Indeed,  
545 it is possible in principle to obtain some amplification with the primers of a segment even when it is  
546 absent if the primers can hybridize at a low rate with other segments or plant DNA sequences. In

547 order to establish threshold Ct beyond which each of segments C, N or U4 could be considered  
548 with confidence as absent, we performed qPCRs on samples of plants which were agro-inoculated  
549 with all FBNSV segments but the focal one. To this end, we agro-inoculated plants with FBNSV<sup>N</sup>,  
550 FBNSV<sup>U4</sup> or FBNSV<sup>C</sup>, following the procedures described earlier. These plants were grown for 21  
551 days in growth chambers under a 13/11 h day/night photoperiod at a temperature of 26/20°C. The  
552 total DNA of plants was then extracted and the presence of segments C, N and U4 was tested in  
553 each plant through qPCR. We could thus obtain the amplification level produced by the primers of  
554 a segment from plants in which the segment was absent. We then calculated for each segment the  
555 lower tolerance threshold such that 95% of potential future samples would have higher values with  
556 a 95% probability (39, 40). These thresholds were 30.37, 31.09 and 28.27 for segments C, N and  
557 U4, respectively (see SI for details on these calculations).

558

#### 559 **iv. Preparation of aphid midguts and Fluorescence in situ hybridization (FISH)**

560

561 We previously showed that, when acquired from a plant infected with FBNSV<sup>complete</sup>, the eight  
562 segments of FBNSV co-localized in peri-nuclear cytoplasmic granules in cells of the anterior midgut  
563 (AMG) of aphid vectors *A. pisum* (27). Here, we specifically labeled the pairs of sequentially  
564 acquired segments (segment C then U4 or N then U4) to determine whether they co-localize in the  
565 AMG cells of their vector. Such eventual co-localization of the two sequentially acquired segments  
566 would indicate that the FBNSV genome reconstitution can occur at early stages of accumulation in  
567 aphid midguts.

568

569 Organ preparation and labeling were performed as detailed in (27). Because 3 days of AAP do not  
570 allow sufficient accumulation of viruses into the vector AMG for visualization, aphids were placed  
571 on plants during 7 days for the first and the second acquisition steps. After the first AAP, aphids  
572 were placed on healthy plants in order to purge viral particles from their gut; in order to limit potential  
573 depletion of the first set of segments, this purge period was limited to a single day. The second  
574 AAP was followed by a 24 h purging period by feeding aphids on water through a Parafilm  
575 membrane to eliminate the virus present in the lumen of the AMG. Then, purged aphids were  
576 dissected. AMGs were soaked in 1X phosphate-buffered saline (PBS; pH 7.4) and fixed for 20 min  
577 in 4% paraformaldehyde (PFA) prepared in 1X PBS. To stop the fixation reaction, AMGs were  
578 incubated in 0.1 M glycine pH 7.4 for 15 min. Organs were washed twice (10 min each) in 1X PBS  
579 and an additional incubation in 30 % H<sub>2</sub>O<sub>2</sub> solution for 20 min was performed to discolor AMGs  
580 and improve the posterior FISH treatment. Finally, AMGs were kept in 1X PBS at 4°C until used for  
581 a maximum storage time of 3 weeks.

582

583 FISH was performed using fluorescent probes specific to segments C, N or U4 prepared as  
584 described in (41). Amplified coding sequences of segments C and N were labeled with Alexa fluor  
585 468 (red) and those for segment U4 were labeled with Alexa fluor 488 (green). Dissected AMGs  
586 kept in 1X PBS were washed three times (5 min each) in hybridization buffer (20 mM Tris-HCl, pH  
587 8, 0.9 M NaCl, 0.01% SDS and 2.7 M of urea that replaced the 30% deionized formamide used in  
588 (41)), before an overnight incubation at 37°C with the segment specific probes (diluted 1/30 in  
589 hybridization buffer). Labeling was stopped by three washes (5 min each) in the hybridization buffer  
590 and then AMGs were placed in a 1X PBS solution. Samples were mounted on microscope slides  
591 in a Vectashield antifade-mounting medium (Vector Laboratories) that contains DAPI for nuclei  
592 staining, and AMGs were observed using the Zeiss LSM700 confocal microscope equipped with a  
593 63X objective.

594

#### 595 **v. Data analysis**

596

597 Based on the Ct threshold values defined above, we determined whether each segment was  
598 present or absent from each recipient plant. In all cases but one, both technical replicates of each  
599 sample were either above or below the threshold. The only exception concerned segment C of one  
600 sample that we conservatively considered as absent.

601  
602 All asymptomatic plants were considered non-infected. All plants showing symptoms were qPCR  
603 processed and if any of the C, N, or U4 segments was present in the sample we considered the  
604 plant as infected. When all three segments were present in recipient plants we considered that a  
605 complete infection was reconstituted.

606  
607 To investigate whether transmission modalities (parallel or sequential) affected the probability of  
608 reconstitution (P(R)) or infection (P(I)), we ran GLMMs considering that P(R) and P(I) were  
609 binomially distributed with logit-distributed errors, transmission modality and segments involved as  
610 fixed factors, and experimental replicate as a random factor. Spacing time was added as a  
611 covariate when relevant. We used the lme4 package in R to run these analyses. Figures were  
612 drawn using JMP 13.2.1. The data and R scripts for these analyses can be found at  
613 <https://doi.org/10.57745/EH9KRW>.

614  
615

### 616 **Acknowledgments**

617

618 This work was funded by ANR Reassort ANR-20-CE02-0016 and ANR Nanovirus ANR-18-CE92-  
619 0028. MY and SB acknowledge support from INRAE dpt. SPE, YM from CNRS and IRD, and JLZ  
620 from IRD. The authors acknowledge valuable technical help provided by Ms Sophie Le Blaye. Many  
621 thanks to Bernard Francq and Thomas Mathew for their insights on tolerance intervals.

622

623

### 624 **References**

625

- 626 1. A. Lucía-Sanz, S. Manrubia, Multipartite viruses: adaptive trick or evolutionary treat? *npj*  
627 *Systems Biology and Applications* **3** (2017).
- 628 2. S. Ojosnegros, *et al.*, Viral genome segmentation can result from a trade-off between genetic  
629 content and particle stability. *PLOS Genetics* **7**, e1001344 (2011).
- 630 3. A. Sicard, Y. Michalakakis, S. Gutiérrez, S. Blanc, The strange lifestyle of multipartite viruses.  
631 *PLOS Pathogens* **12**, e1005819 (2016).
- 632 4. J. Iranzo, S. C. Manrubia, Evolutionary dynamics of genome segmentation in multipartite  
633 viruses. *Proc Biol Sci* **279**, 3812–3819 (2012).
- 634 5. R. Gallet, *et al.*, Small bottleneck size in a highly multipartite virus during a complete infection  
635 cycle. *Journal of Virology* **92**, e00139-18 (2018).
- 636 6. B. Gronenborn, Nanoviruses: genome organisation and protein function. *Veterinary*  
637 *Microbiology* **98**, 103–109 (2004).
- 638 7. A. Lal, *et al.*, Nanovirus disease complexes: an emerging threat in the modern era. *Frontiers in*  
639 *Plant Science* **11** (2020).
- 640 8. A. Sicard, *et al.*, Gene copy number is differentially regulated in a multipartite virus. *Nature*  
641 *Communications* **4**, DOI: 10.1038/ncomms3248 (2013).
- 642 9. M. Mansourpour, *et al.*, Effects of an alphasatellite on life cycle of the nanovirus Faba bean  
643 necrotic yellows virus. *Journal of Virology* **96**, e01388-21 (2021).
- 644 10. Z. Hu, *et al.*, Genome segments accumulate with different frequencies in *Bombyx mori*  
645 bidensovirus. *Journal of Basic Microbiology* **56**, 1338–1343 (2016).

- 646 11. B. Wu, M. P. Zwart, J. A. Sánchez-Navarro, S. F. Elena, Within-host evolution of segments  
647 ratio for the tripartite genome of Alfalfa Mosaic Virus. *Scientific Reports* **7**, 5004 (2017).
- 648 12. A. Sicard, *et al.*, A multicellular way of life for a multipartite virus. *eLife* **8**, e43599 (2019).
- 649 13. M. Betancourt, A. Fereres, A. Fraile, F. García-Arenal, Estimation of the effective number of  
650 founders that initiate an infection after aphid transmission of a multipartite plant virus. *Journal*  
651 *of Virology* **82**, 12416–12421 (2008).
- 652 14. Y. Michalakis, S. Blanc, The curious strategy of multipartite viruses. *Annual Review of Virology*  
653 **7**, 203–218 (2020).
- 654 15. A. Franz, K. M. Makkouk, H. J. Vetten, Host range of faba bean necrotic yellows virus and  
655 potential yield loss in infected faba bean. *Phytopathologia Mediterranea* **36**, 94–103 (1997).
- 656 16. I. Grigoras, *et al.*, Genome diversity and evidence of recombination and reassortment in  
657 nanoviruses from Europe. *Journal of General Virology* **95**, 1178–1191 (2014).
- 658 17. A. D. Abraham, *et al.*, Two distinct nanovirus species infecting faba bean in Morocco. *Arch Virol*  
659 **155**, 37–46 (2010).
- 660 18. M. Lotfipour, S. A. A. Behjatnia, M. Dall'Ara, C. Ratti, The full-length genome characterization  
661 and diversity of faba bean necrotic stunt virus in Iran. *Eur J Plant Pathol* **157**, 239–250 (2020).
- 662 19. T. Timchenko, Infectivity of nanovirus DNAs: induction of disease by cloned genome  
663 components of Faba bean necrotic yellows virus. *Journal of General Virology* **87**, 1735–1743  
664 (2006).
- 665 20. I. Grigoras, *et al.*, Nanovirus DNA-N encodes a protein mandatory for aphid transmission.  
666 *Virology* **522**, 281–291 (2018).
- 667 21. Y. Michalakis, S. Blanc, Editorial overview: Multicomponent viral systems. *Current Opinion in*  
668 *Virology* **33**, vi–ix (2018).
- 669 22. A. Y. S. Chen, G. P. Walker, D. Carter, J. C. K. Ng, A virus capsid component mediates virion  
670 retention and transmission by its insect vector. *Proceedings of the National Academy of*  
671 *Sciences* **108**, 16777–16782 (2011).
- 672 23. D. J. F. Brown, W. M. Robertson, D. L. Trudgill, Transmission of viruses by plant nematodes.  
673 *Annu. Rev. Phytopathol.* **33**, 223–249 (1995).
- 674 24. K. E. Mauck, C. M. De Moraes, M. C. Mescher, Deceptive chemical signals induced by a plant  
675 virus attract insect vectors to inferior hosts. *Proceedings of the National Academy of Sciences*  
676 **107**, 3600–3605 (2010).
- 677 25. A. Sicard, *et al.*, Circulative nonpropagative aphid transmission of nanoviruses: an  
678 oversimplified view. *J Virol* **89**, 9719–26 (2015).
- 679 26. J. Di Mattia, *et al.*, Route of a multipartite Nanovirus across the body of its aphid vector. *J Virol*  
680 **94**, e01998-19 (2020).
- 681 27. J. D. Mattia, *et al.*, Route of a multipartite nanovirus across the body of its aphid vector. *Journal*  
682 *of Virology* **94** (2020).

- 683 28. A. W. E. Franz, F. van der Wilk, M. Verbeek, A. M. Dullemans, J. F. J. M. van den Heuvel,  
684 Faba bean necrotic yellows virus (Genus Nanovirus) requires a helper factor for its aphid  
685 transmission. *Virology* **262**, 210–219 (1999).
- 686 29. B. Kassanis, D. A. Govier, New evidence on the mechanism of aphid transmission of potato C  
687 and potato aucuba mosaic viruses. *J Gen Virol* **10**, 99–101 (1971).
- 688 30. B. Kassanis, D. A. Govier, The role of the helper virus in aphid transmission of potato aucuba  
689 mosaic virus and potato virus C. *J Gen Virol* **13**, 221–228 (1971).
- 690 31. M. C. Y. Lung, T. P. Pirone, Studies on the reason for differential transmissibility of cauliflower  
691 mosaic virus isolates by aphids. *Phytopathology* **63**, 910 (1973).
- 692 32. G. Lu, *et al.*, Tenuivirus utilizes its glycoprotein as a helper component to overcome insect  
693 midgut barriers for its circulative and propagative transmission. *PLOS Pathogens* **15**, e1007655  
694 (2019).
- 695 33. Y. Z. A. Gaafar, H. Ziebell, Aphid transmission of nanoviruses. *Archives of Insect Biochemistry  
696 and Physiology* **104**, e21668 (2020).
- 697 34. S. Watanabe, A. Bressan, Tropism, compartmentalization and retention of banana bunchy top  
698 virus (Nanoviridae) in the aphid vector *Pentalonia nigronervosa*. *J Gen Virol* **94**, 209–219  
699 (2013).
- 700 35. I. Grigoras, *et al.*, Reconstitution of authentic Nanovirus from multiple cloned DNAs. *Journal of  
701 Virology* **83**, 10778–10787 (2009).
- 702 36. R. Froissart, Y. Michalakis, S. Blanc, Helper component-transcomplementation in the vector  
703 transmission of plant viruses. *Phytopathology* **92**, 576–579 (2002).
- 704 37. J. Di Mattia, *et al.*, Co-acquired nanovirus and geminivirus exhibit a contrasted localization  
705 within their common aphid vector. *Viruses* **12**, 299 (2020).
- 706 38. R. Gallet, F. Fabre, Y. Michalakis, S. Blanc, The number of target molecules of the amplification  
707 step limits accuracy and sensitivity in ultradeep-sequencing viral population studies. *Journal of  
708 Virology* **91**, e00561-17 (2017).
- 709 39. G. Sharma, T. Mathew, One-sided and two-sided tolerance intervals in general mixed and  
710 random effects models using small-sample asymptotics. *Journal of the American Statistical  
711 Association* **107**, 258–267 (2012).
- 712 40. B. G. Francq, D. Lin, W. Hoyer, Confidence, prediction, and tolerance in linear mixed models.  
713 *Statistics in Medicine* **38**, 5603–5622 (2019).
- 714 41. M.-S. Vernerey, E. Pirolles, S. Blanc, A. Sicard, Localizing genome segments and protein  
715 products of a multipartite virus in host plant cells. *Bio Protoc* **9**, e3443 (2019).
- 716  
717



718 **Figure Legends**

719

720

721 **Figure 1. Schematic of the experimental design of parallel and sequential transmission**  
722 **modes.** Details of the two transmission modes are schematized as well as their expected outcome  
723 using infections lacking segments C and U4 as an example. For sequential transmissions we show  
724 only the case where aphids first fed on plants infected with FBNSV<sup>C-</sup> and then fed on plants infected  
725 with FBNSV<sup>U4-</sup>. The age of test plants, the status and age of infections, the duration of acquisition  
726 access periods (AAP) and of inoculation access periods (IAP) are indicated. The parallel and  
727 sequential with no time spacing treatments were performed during Experiment 1, the sequential  
728 with time spacing treatments were performed during Experiment 2 (the sequential with no time  
729 spacing treatment was independently performed in both experiments). Each experiment was  
730 replicated twice. As mentioned in the Materials and Methods, the parallel transmission mode  
731 implies separate acquisition and inoculation of segments, whereas the sequential transmission  
732 mode implies temporally separated acquisition of segments but may allow concomitant inoculation.

733

734 **Figure 2. Infection and full genome reconstitution as a function of the transmission mode.**  
735 Proportion of infected plants (blue) and plants where all three investigated segments are found  
736 (reconstitution, red) as a function of the transmission mode (parallel vs. sequential) and the  
737 segments involved (parallel: only U4 and C; sequential: either U4 and C or N and U4; the order of  
738 the segments in the labels indicates the order of infected plants on which the aphids fed). For  
739 sequential transmission and for a given pair of segments and order, we provide the results from  
740 the two different experiments, “parallel vs sequential” (Exp 1) and “sequential with spacing in time”  
741 (Exp 2), using for the latter only the 0 spacing time treatments. The two experimental replicates (1,  
742 2) are shown for each case, and the error bars are  $\pm 1$  s.e. of the mean. Sample size varies from  
743 20 to 23 (see table 1).

744

745

746 **Figure 3. Infection and full genome reconstitution as a function of time spacing between**  
747 **sequential acquisitions.** Proportion of infected plants (blue) and plants in which all three  
748 investigated segments are found (reconstitution, red) as a function of time spacing (top x-axis,  
749 expressed in days) for the different segments and order combinations. The two experimental  
750 replicates are shown for each case, and the error bars are  $\pm 1$  s.e. of the mean. Sample size varies  
751 from 17 to 24 (see table 1).

752

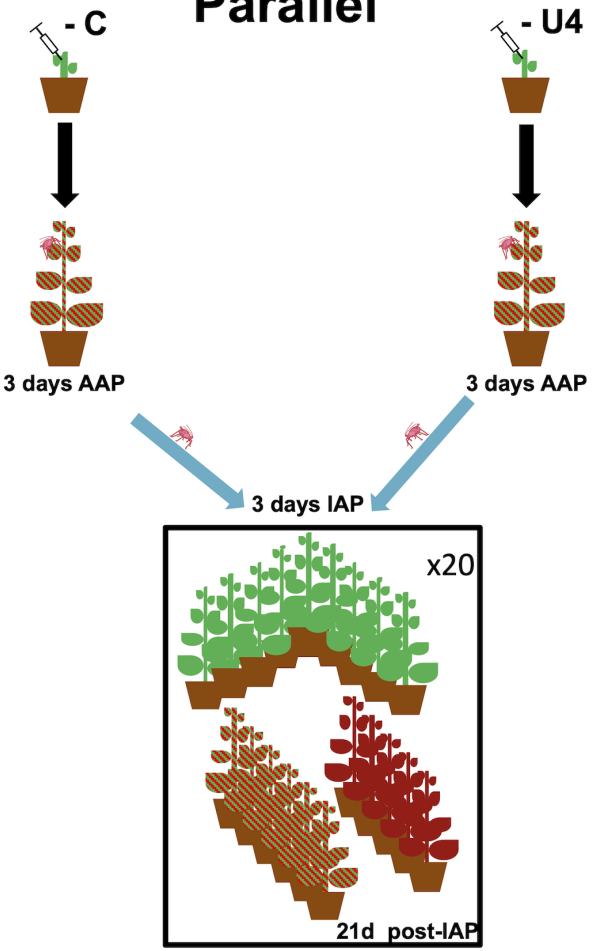
753

754 **Figure 4. Localization of sequentially acquired DNA segments of FBNSV in aphid anterior**  
755 **midgut (AMG) cells.** Viral DNA is labeled by FISH in anterior midgut (AMG) cells of viruliferous  
756 aphids and observed by confocal microscopy. The green probe targets segment U4 and the red  
757 probes target either C or N in the corresponding panels. The accumulation of FBNSV DNA was  
758 similarly revealed in all observed cells (>10 cells per midgut) from 16, 10, 6 and 14 viruliferous  
759 aphids for (A), (B), (C) and (D), respectively. A representative image of each case is shown to  
760 illustrate the results. Panels A and B represent the sequential acquisition of segments C/U4, and  
761 C and D that of segments N/U4, in the indicated sequential order. The white arrows show two foci  
762 containing both C and U4 segments. All images correspond to single optical sections. Cell nuclei  
763 are stained with DAPI (4,6-diamidino-2-phenylindole; blue). The scale bar represents 25  $\mu\text{m}$ .

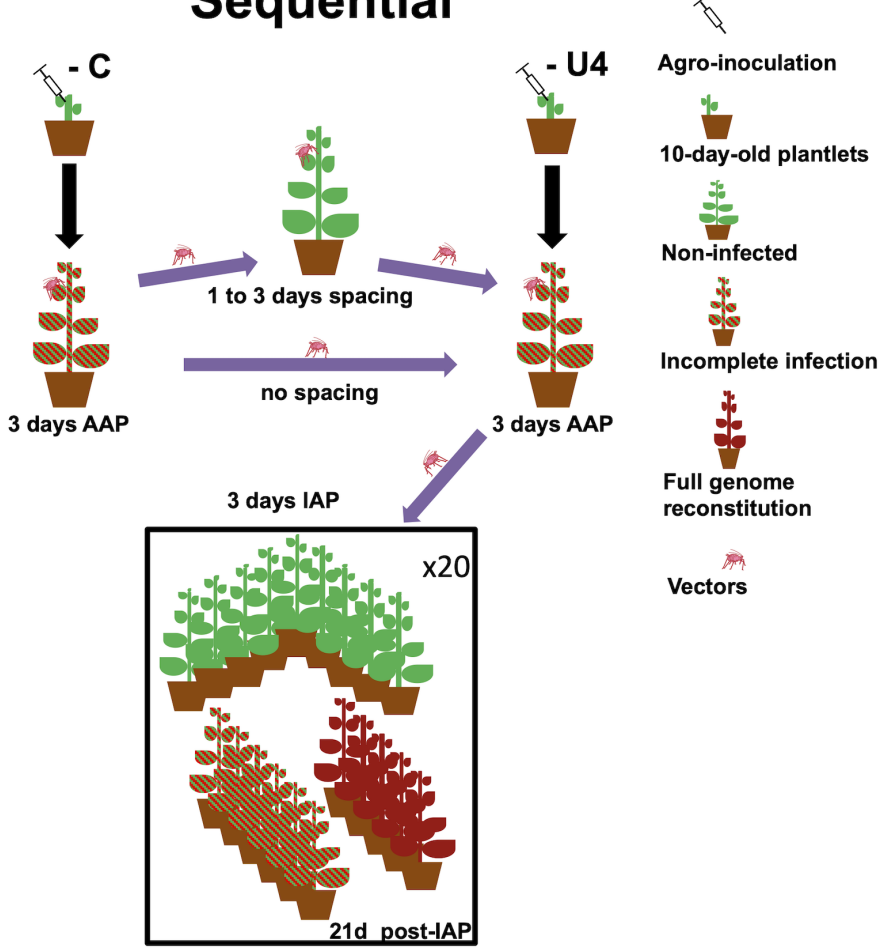
764

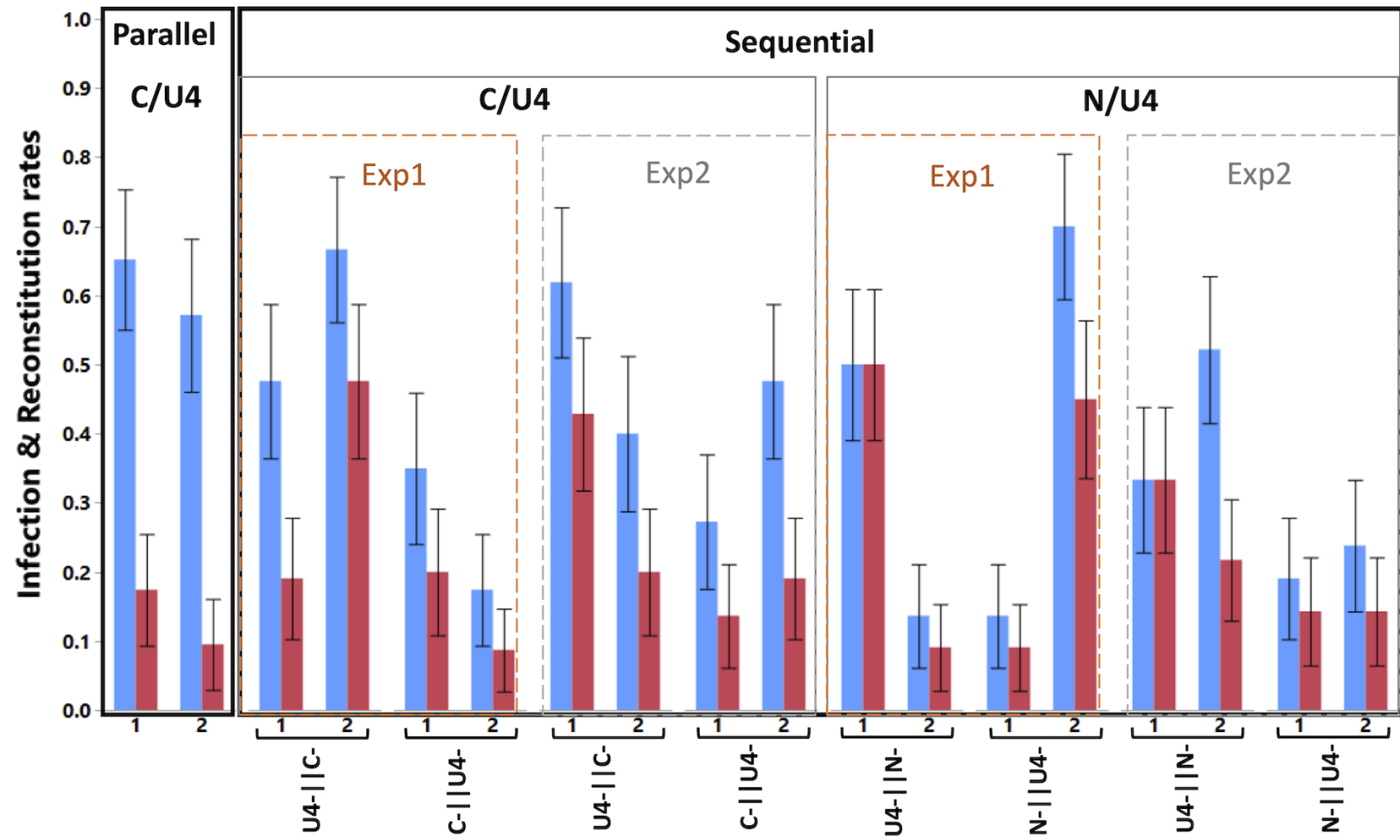
765

# Parallel

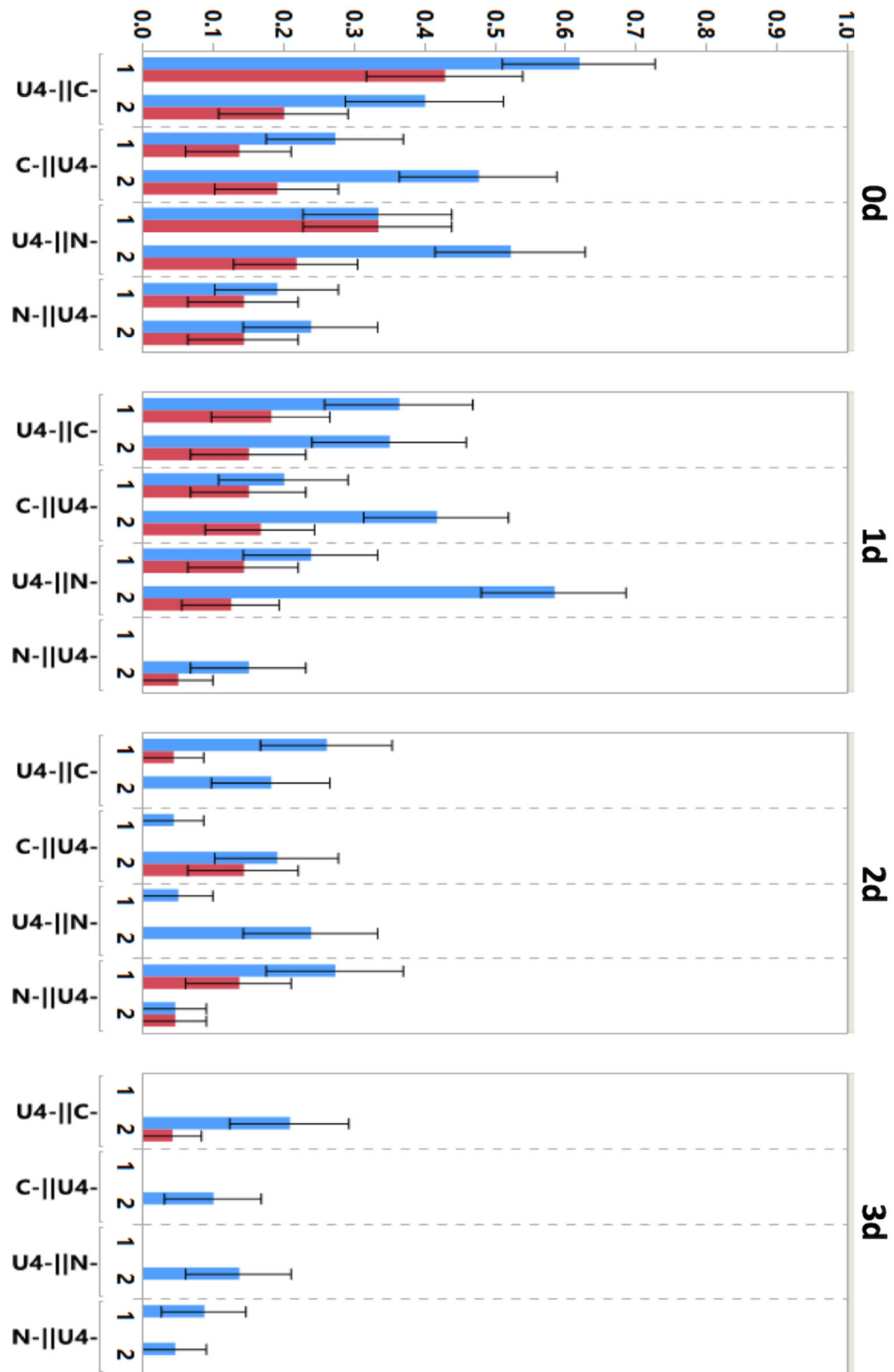


# Sequential





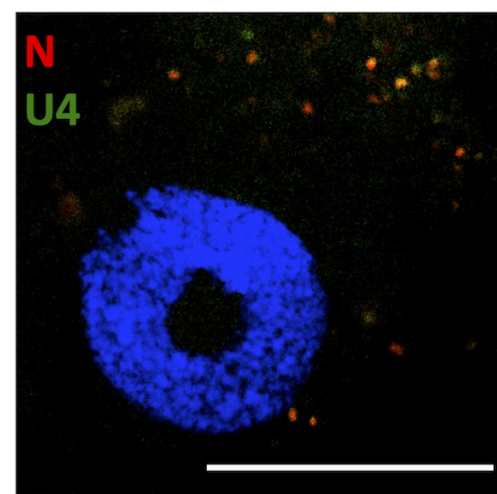
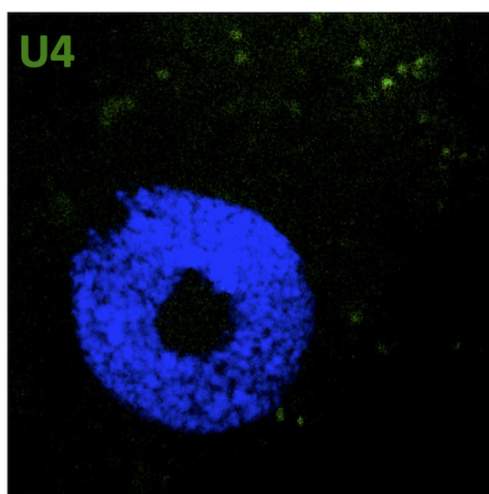
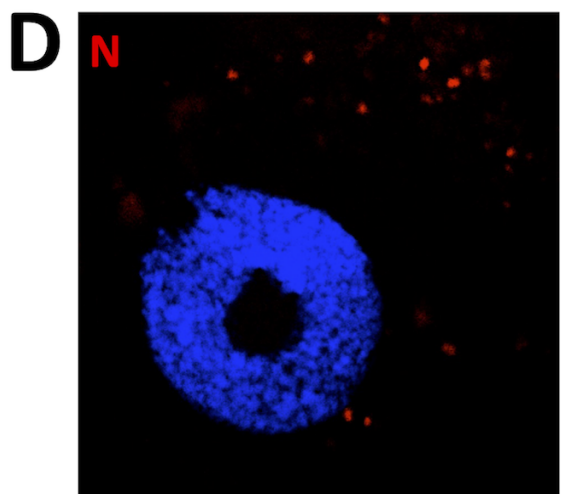
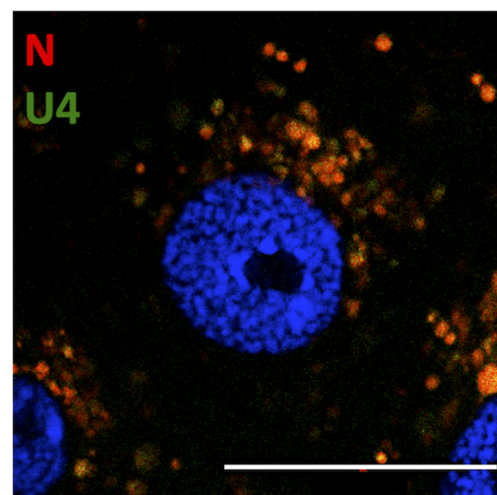
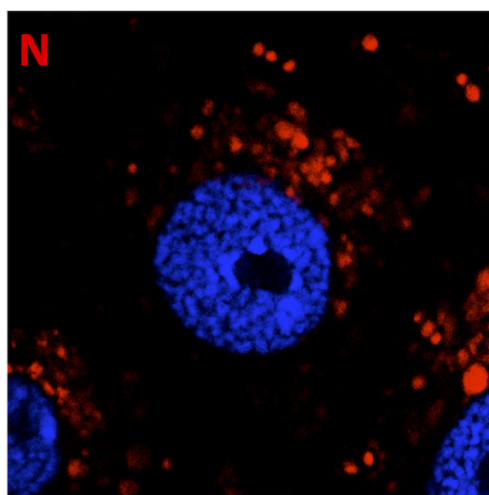
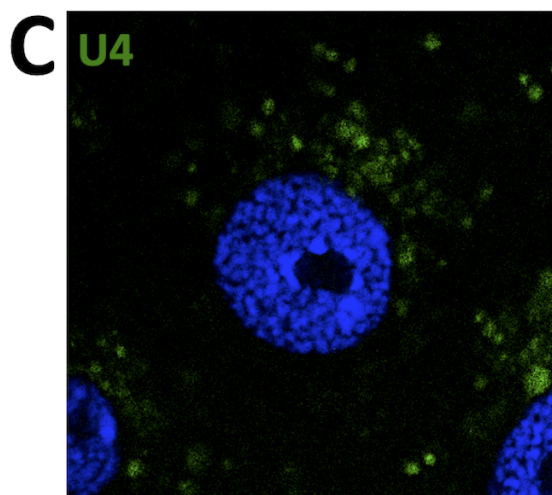
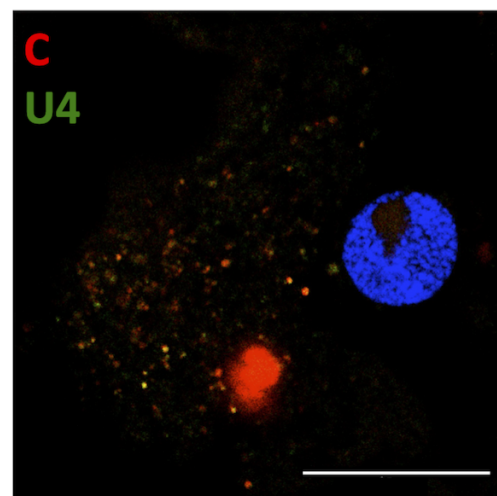
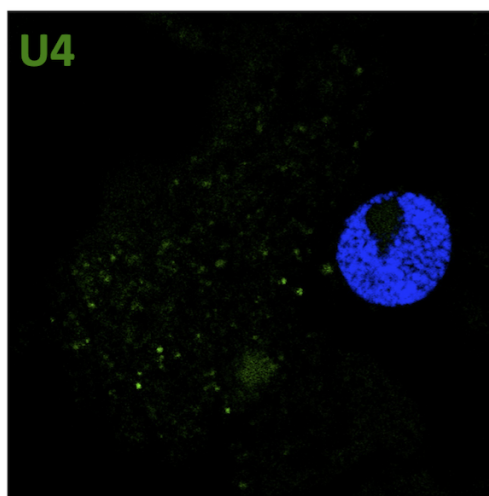
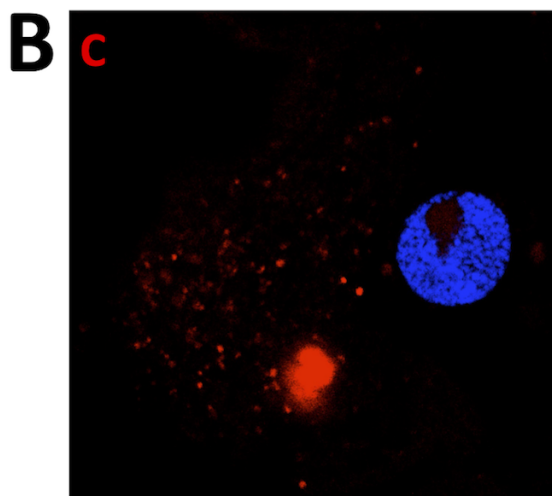
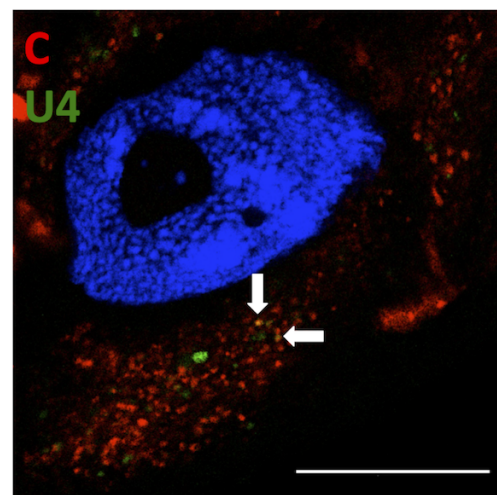
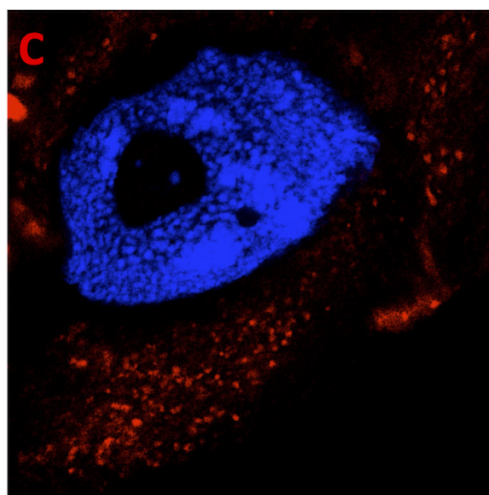
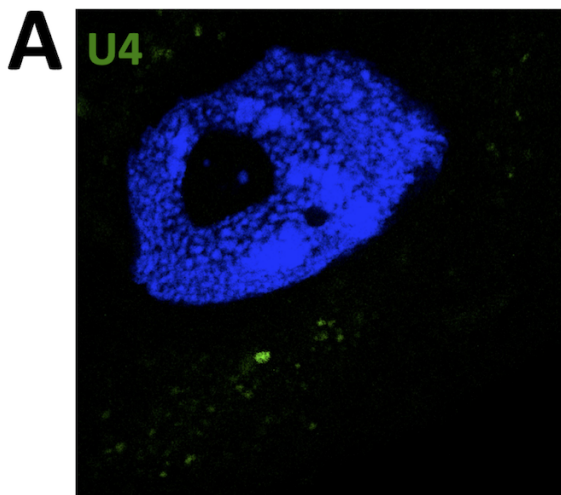
### Infection & Reconstitution rates



First segment acquired

Second segment acquired

Merge



**Table 1.** Number of inoculated plants for each experiment, transmission mode, segments combination and replicate.

		Number of inoculated plants									
	Experiment	(i)					(ii)				
	Transmission mode	Parallel	Sequential				Sequential				
	Combinations		1	2	3	4	1	2	3	4	
Spacing time (days)	Replicate	a b	a b	a b	a b	a b	a b	a b	a b	a b	
0		20 23	20 23	21 21	22 20	22 22	22 21	21 20	21 21	21 23	
1							20 24	22 20	21 20	21 24	
2							23 21	23 22	22 22	20 21	
3							17 20	21 24	23 22	23 22	

Combinations:

- (1). Aphids fed on FBNSV<sup>C-</sup> (plants without segment C) infected plants and then on FBNSV<sup>U4-</sup> (plants without segment U4) infected plants
- (2). Aphids fed on FBNSV<sup>U4-</sup> infected plants then on FBNSV<sup>C-</sup> infected plants
- (3). Aphids fed on FBNSV<sup>N-</sup> (plants without segment N) infected plants then on FBNSV<sup>U4-</sup> infected plants
- (4). Aphids fed on FBNSV<sup>U4-</sup> infected plants then on FBNSV<sup>N-</sup> infected plants

Median/mean sample size = 21/21.5