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

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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

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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^{C-}, FBNSV^{N-} or FBNSV^{U4-}, 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 **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

417 In this study we inoculated ten-day-old faba bean plantlets (*Vicia faba* cv. Sevilla; Vilmorin) with
418 FBNSV^{complete}, FBNSV^{C-}, FBNSV^{N-} or FBNSV^{U4-} agrobacteria solutions (strain COR308), as earlier
419 described (35). While FNSV^{complete} 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^{C-}, FBNSV^{N-} or FBNSV^{U4-}. 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^{N-} and FBNSV^{U4-}, respectively 51.06% (=96/188) and
425 52.81% (=94/178), we could only obtain 13.08% (=56/428) infected plants with FBNSV^{C-}.

426
427

427 **ii. Aphid transmission**

428
429

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

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^{C-} and two FBNSV^{U4-} 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^{C-} and two FBNSV^{U4-} 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^{C-} or FBNSV^{U4-}) 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^{C-} then FBNSV^{U4-} (C-||U4-), (2)
470 FBNSV^{U4-} then FBNSV^{C-} (U4-||C-), (3) FBNSV^{N-} then FBNSV^{U4-} (N-||U4-) and (4) FBNSV^{U4-} then
471 FBNSV^{N-} (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 μ L micropipette tip and added 100 μ 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 μ 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 μ 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 μ L of the 2X qPCR Mastermix, 0.3 or 0.5 μ M primers (0.3 μ M final for segment C and 0.5 μ M for
536 segments N and U4 – specific primers for FBNSV segments were described in (6)), complemented
537 at 8 μ L with H₂O. Two μ L of DNA sample were added to a final mix of 10 μ 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^N,
550 FBNSV^{U4} or FBNSV^C, 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^{complete}, 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 % H₂O₂ 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

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617

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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. Piroles, 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^{C-} and then fed on plants infected
725 with FBNSV^{U4-}. 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 ± 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 ± 1 s.e. of the mean. Sample size varies
751 from 17 to 24 (see table 1).

752

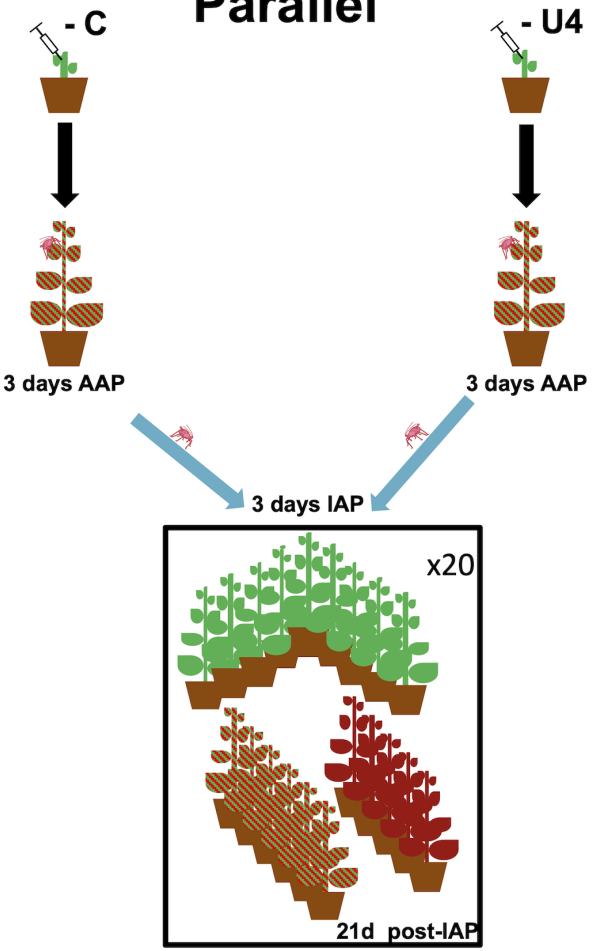
753

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 μm .

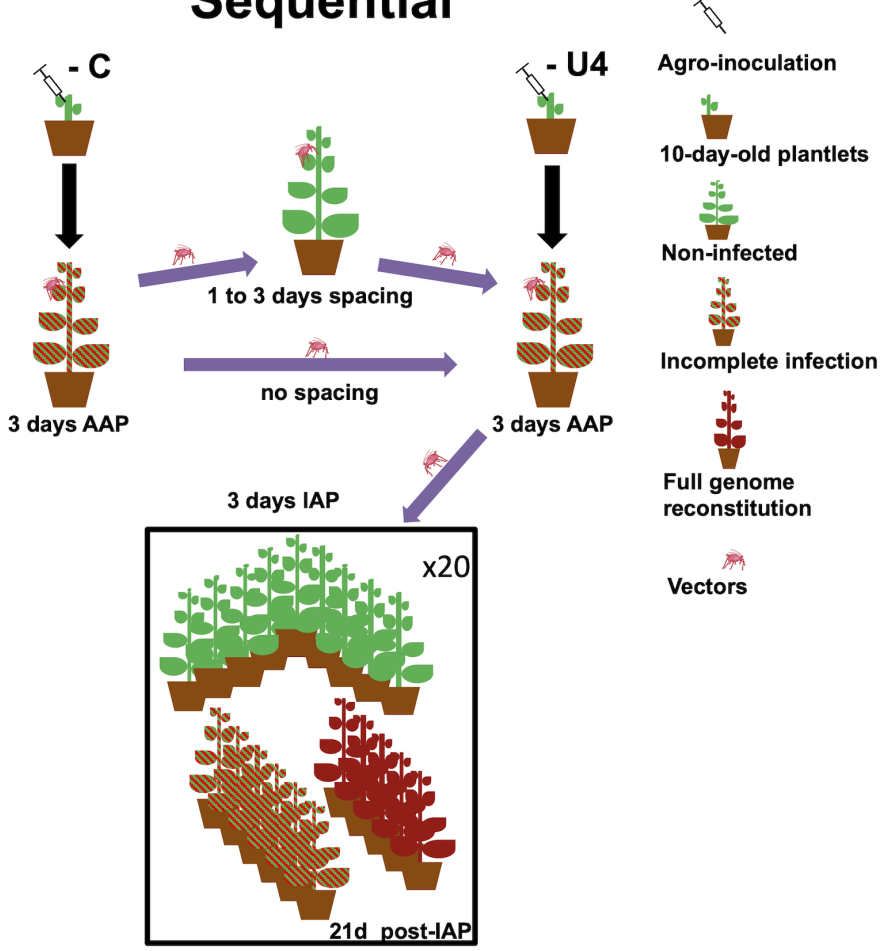
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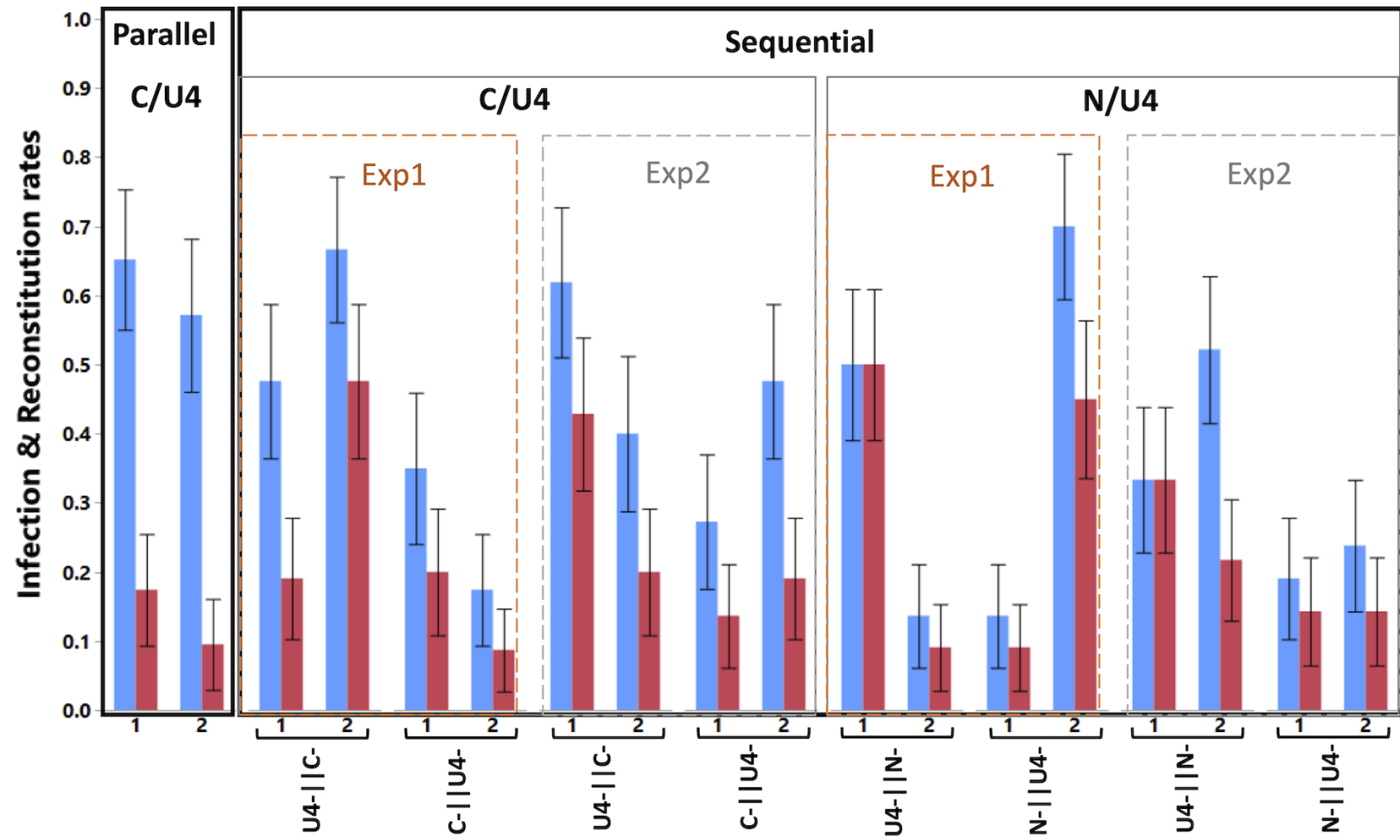
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Parallel

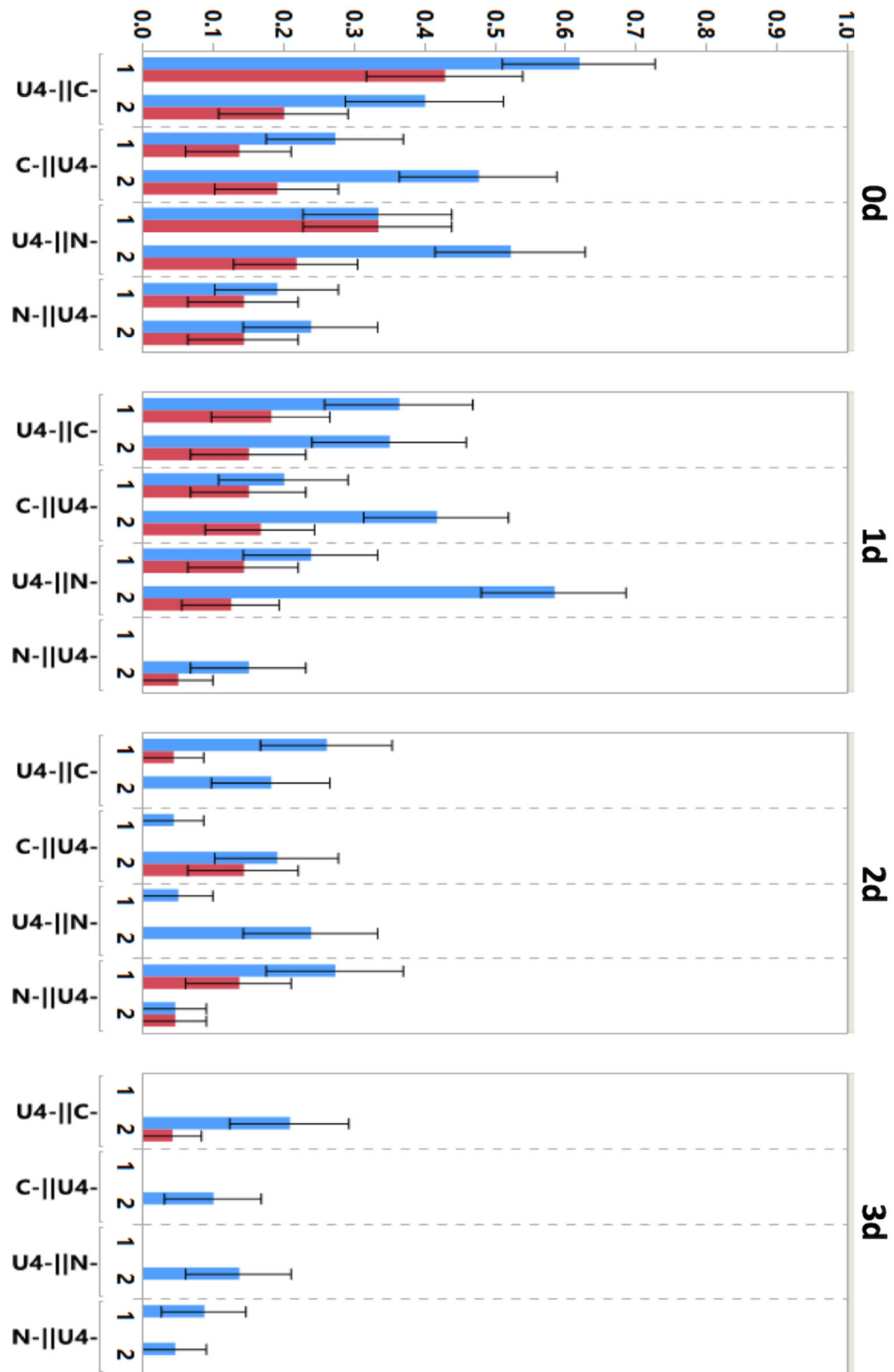


Sequential





Infection & Reconstitution rates



First segment acquired

Second segment acquired

Merge

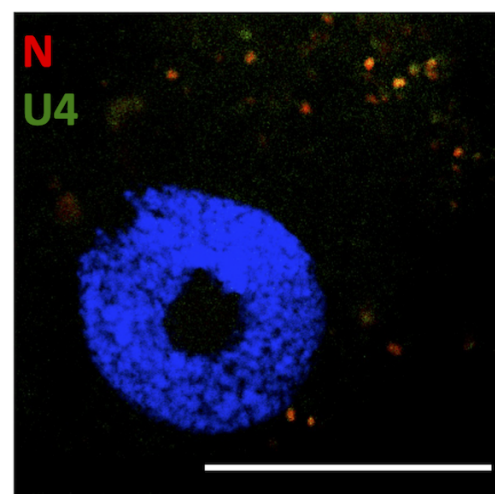
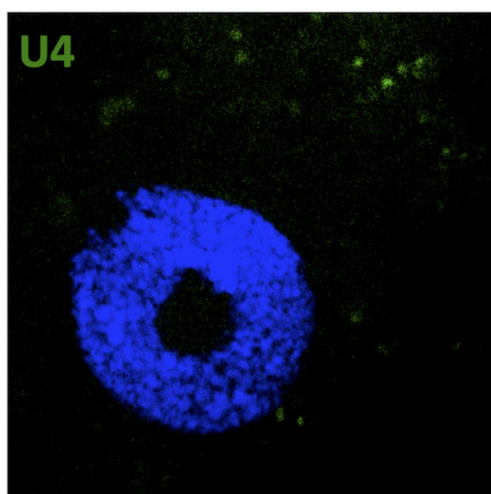
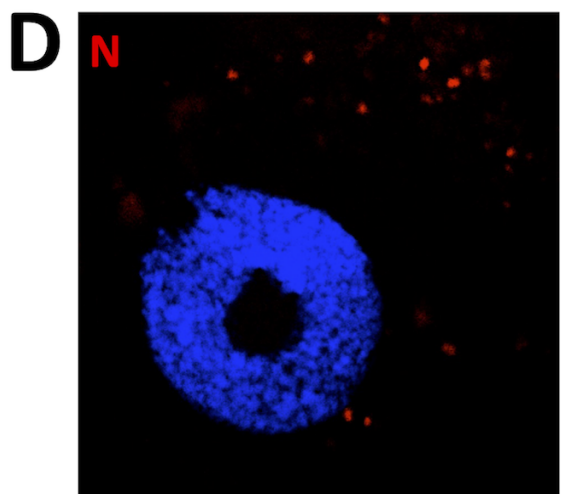
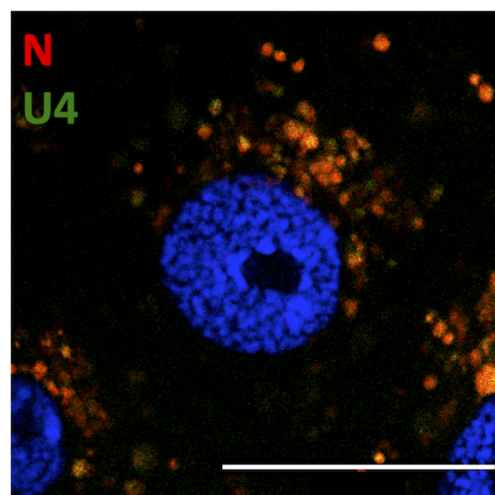
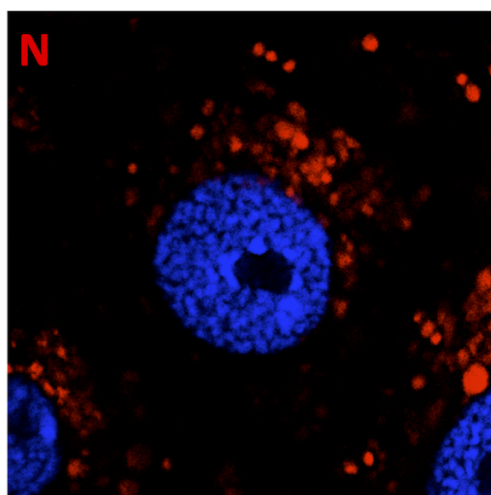
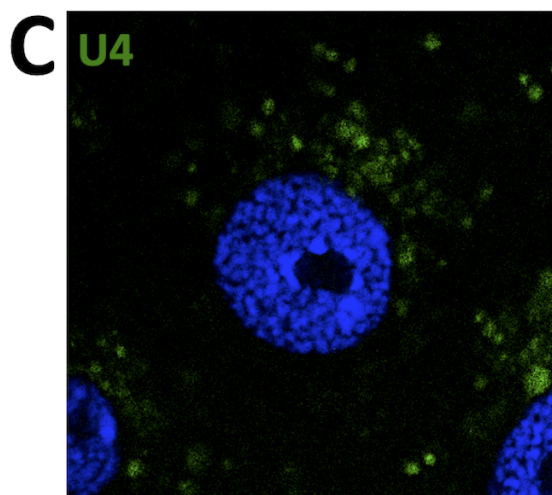
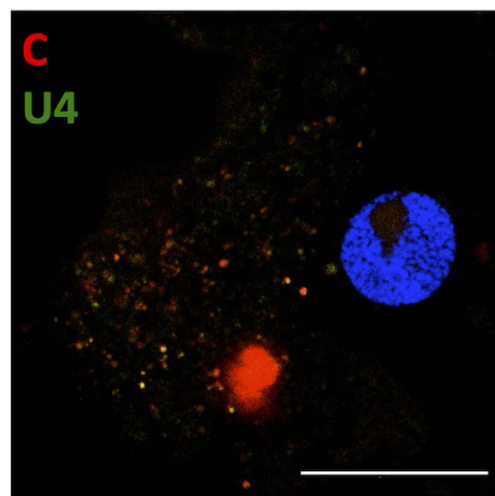
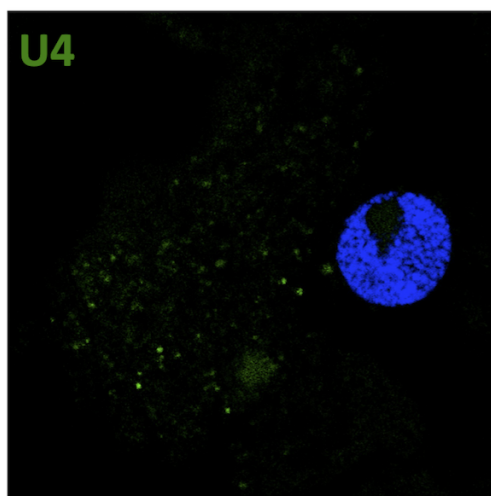
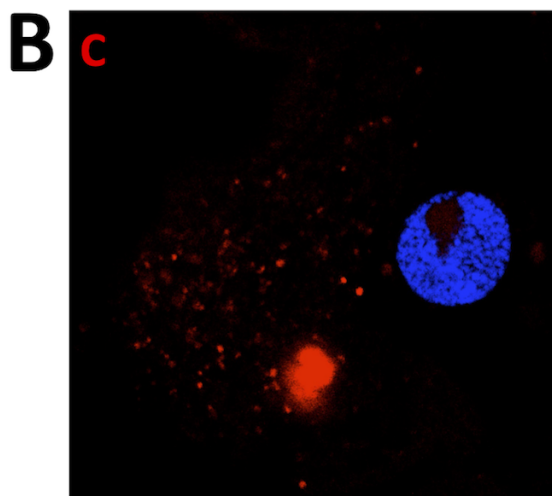
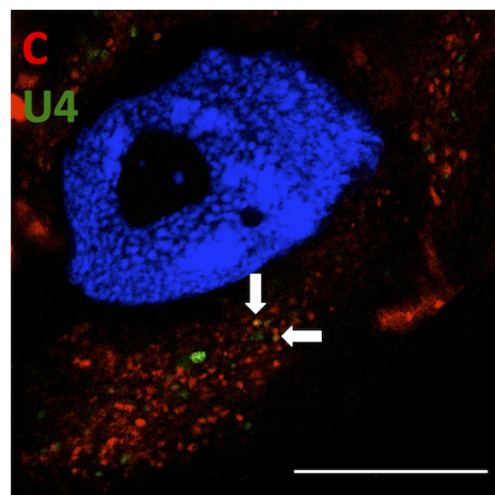
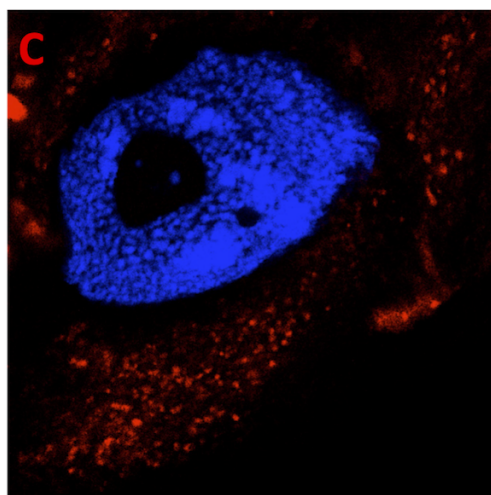
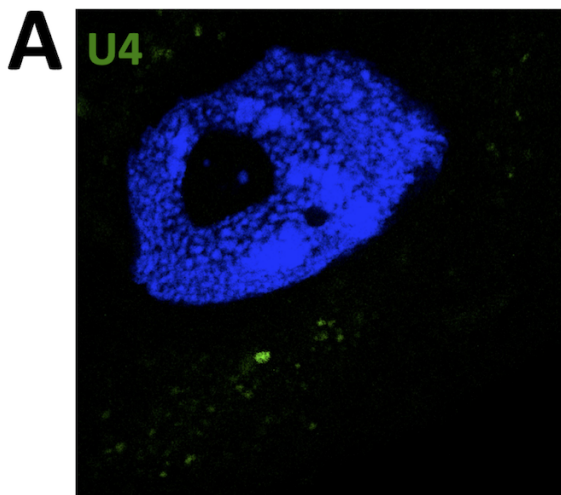


Table 1. Number of inoculated plants for each experiment, transmission mode, segments combination and replicate.

		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^{C-} (plants without segment C) infected plants and then on FBNSV^{U4-} (plants without segment U4) infected plants
- (2). Aphids fed on FBNSV^{U4-} infected plants then on FBNSV^{C-} infected plants
- (3). Aphids fed on FBNSV^{N-} (plants without segment N) infected plants then on FBNSV^{U4-} infected plants
- (4). Aphids fed on FBNSV^{U4-} infected plants then on FBNSV^{N-} infected plants

Median/mean sample size = 21/21.5