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2 **Main Manuscript for**

3 The genome of a bunyavirus cannot be defined at the level of the viral particle but only
4 at the scale of the viral population

5

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23

24 **This PDF file includes:**

25 Main Text
26 Figures Legends
27 (Figures 1-4, Supplemental Table S1 and S2, Figure S1 and Movie S1 are supplied as separate
28 files)
29

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36

37 **Abstract**

38

39 Bunyaviruses are enveloped negative or ambisense single stranded RNA viruses with a genome
40 divided into several segments. The canonical view depicts each viral particle packaging one copy
41 of each genomic segment in one polarity named the viral strand. Several opposing observations
42 revealed non-equal ratios of the segments, uneven number of segments per virion, and even
43 packaging of viral complementary strands. Unfortunately, these observations result from studies
44 often addressing other questions, on distinct viral species, and not using accurate quantitative
45 methods. Hence, what RNA segments and strands are packaged as the genome of any
46 bunyavirus remains largely ambiguous. We addressed this issue by first investigating the virion
47 size distribution and RNA content in populations of the tomato spotted wilt virus (TSWV) using
48 microscopy and tomography. These revealed heterogeneity in viral particle volume and amount
49 of RNA content, with a surprising lack of correlation between the two. Then, the ratios of all
50 genomic segments and strands were established using RNA sequencing and RT-qPCR. Within
51 virions, both plus and minus strands (but no mRNA) are packaged for each of the three L, M and
52 S segments, in reproducible non-equimolar proportions determined by those in total cell
53 extracts. These results show that virions differ in their genomic content but together build up a
54 highly reproducible genetic composition of the viral population. This resembles the genome
55 formula described for multipartite viruses, with which some species of the order *Bunyavirales*
56 may share some aspects of the way of life, particularly emerging properties at a supra-virion
57 scale.

58

59 **Significance Statement**

60

61 Bunyaviruses infect animals, plants, fungi, and protists. Despite their importance for global
62 health, fundamental aspects of their biology as basic as the definition of their genome remain
63 elusive. The viral genome consists of several negative or ambisense RNA segments, and virions
64 often miss segments and/or package complementary strands. We formally quantify this
65 heterogeneity on the species *Tomato spotted wilt orthospovirus*. Within individual virus
66 particles, the number, the identity and even the polarity of the segments is widely variable. In
67 contrast, we show that a stable genetic composition is an emerging property of the viral
68 population, each of the RNA segments/polarities accumulating reproducibly at a specific
69 frequency. This resembles the genome formula of multipartite viruses, suggesting that
70 bunyaviruses may also function as multicomponent viral systems.

71

72 **Main Text**

73

74 **Introduction**

75

76 The order *Bunyavirales* was established by the ICTV to contain viral species with 2-8 single-
77 stranded RNA genomic components with negative (sequence complementary to coding strand)
78 or ambisense (coding in both orientations) polarity (1)(2). The order is divided into 14 families
79 that include nearly 500 virus species infecting vertebrates, invertebrates, plants, fungi and
80 protists (2, 3). Many of the well-characterized member species of this order are considered
81 emerging viruses and cause serious diseases impacting human health and agriculture
82 productivity globally (4, 5). Except for the family *Hantaviridae*, all bunyaviruses infecting animals
83 and plants are transmitted by insect or tick vectors in which the virus can also replicate,
84 complicating efforts to control disease spread.

85 Despite the importance of this viral order, some very basic aspects of the biology of the most
86 important species remain mysterious. To illustrate this statement, one can consider the very
87 simple question of what the genome of these viruses is, *i.e.* what is effectively packaged and
88 transmitted to new hosts. The canonical view describes the production of membrane-bound
89 glycoproteins that together delineate a spherical particle. Each particle is considered to enclose
90 one of each of the negative or ambisense RNA segments. Packaged together these segments
91 constitute the complete genome that is transmitted to a new host, putatively as a “one is
92 enough” particle. The current terminology resulting from these assumptions describes the virion
93 contained RNAs as viral sense (vRNAs), and the unencapsidated complementary RNAs generated
94 during genome replication as viral complementary sense (vcRNAs). The literature, including the
95 most prominent Virology web sites, is replete with cartoons depicting packaged genomic
96 segments of vRNA complexed with the N protein that interacts with the inner domain of the
97 glycoproteins associated with the membrane (for example see Refs(6, 7)). A few copies of the
98 polymerase, the L protein, are also shown associated with the genomic RNA segments within
99 particles, as this protein is required for transcription of negative vRNA strands prior to protein
100 production.

101 Many experimental findings contradict this dogma (8). First, the viral particles have been
102 reported to be of highly variable size (9, 10) and, in the case of Lacrosse virus (LACV), this

103 particle size variation has been speculated to correlate with RNA content variation (9). Second,
104 some semi-quantitative observations of the relative amounts of the distinct RNA segments in
105 purified virus particles of different *Bunyavirales* species have indicated that they are not equi-
106 molar (11–17), ruling out a presumed efficient packaging of exactly one copy of each segment
107 per virion. Third, in numerous instances such as tomato spotted wilt virus (13) (TSWV),
108 Uukuniemi virus (18) (UUKV) and rift valley fever virus (19, 15) (RVFV), viral complementary
109 sense strands of the S and M RNAs have been convincingly detected within virions, though in
110 low amount not formally quantified. Whether the encapsidation of the vcRNAs is functionally
111 relevant or whether it is an exception or the rule in the order *Bunyavirales* is thus far unclear.
112 Fourth, the possibility of packaging some viral mRNA has been envisaged (13, 15), though
113 experimental evidence appears to plead against it. Finally, the identity of RNA segments and
114 their number within individual virus particles of RVFV has been directly studied via an elegant
115 single molecule *in situ* hybridization approach (15, 17). Distinguishing between vRNAs and
116 vcRNAs polarities (15) or not (17), these two seminal studies very convincingly showed, for the
117 first time with a direct approach, that virions differ in their RNA content, some missing segments
118 whereas others having extra copies (20), and that the majority of virus particles within a
119 population are not able to express the complete viral genetic information. It is interesting to
120 note that the same studies demonstrated that particles containing incomplete segment sets are
121 nevertheless capable of entering host cells; hence, they could putatively have a functional role
122 through complementation. Consistent with this, earlier papers described genetic engineering of
123 artificial segments of UUKV that could not ensure the complete replication cycle but were
124 nevertheless packaged and possibly transmitted to neighboring cells (21, 22). Thus, despite
125 considerable evidence that the packaging of genome segments of bunyaviruses is highly
126 heterogeneous, which RNA species are packaged (segment and polarity), in what proportions,
127 whether these proportions are reproducible at the virus population level and whether the
128 packaging process is non-specific, are still open questions that have never been formally and
129 quantitatively addressed together on a single species.

130 Here, using the plant infecting TSWV, we explore these questions with several complementary
131 quantitative approaches. With few variations, the molecular biology of TSWV is similar to many
132 member species of the order *Bunyavirales* infecting animals (5), with three segments (L, M, and

133 S) of negative (L) or ambisense polarity (M and S). Using (cryo-)electron microscopy and
134 tomography we establish the virus particle size distribution. We further characterize the amount
135 of RNA content of viral particles and demonstrate that RNA content and particle size do not
136 correlate. Using Nanopore direct RNA sequencing we show that both vRNAs and vcRNAs of all
137 three genomic segments are packaged, though in different proportions depending on the
138 segments. In contrast, messenger RNAs are not packaged, not even in minute amounts. The
139 development of a strand-specific Reverse Transcription (RT) qPCR protocol allowed us to
140 thoroughly quantify the number of copies of each segment and polarity in a large number of
141 samples, indicating that each RNA species is packaged in very reproducible proportions across
142 infected plants thereby defining a “genome formula” at the population level. Finally, the
143 genome formula obtained from infected plant total extracts is very similar to that obtained from
144 virus particles isolated from these extracts, formally supporting a non-specific packaging process
145 where all “available” viral genomic RNAs, of both polarities, are packaged with the same
146 efficiency. Together, by demonstrating that the genome is not recapitulated in individual but in
147 a population of virions, our results highlight important issues of the biology of bunyaviruses, at
148 least of TSWV, that are reminiscent of the biology of multipartite viruses.

149

150 **Results**

151

152 **Virus particle size and RNA content are highly variable and do not correlate**

153 To accurately estimate the diameter of TSWV virions, we recorded cryo-electron microscopy
154 (cryo-EM) images of the frozen-hydrated purified virus preparation (Figure 1A). With this
155 observation method, the sample is immediately frozen/vitrified in a cryogenic liquid and imaged
156 at very low temperature, avoiding distortion of the morphology of biological objects. From cryo-
157 EM images, we estimated the diameter of 4861 purified TSWV particles, which appear as near
158 perfect spheres. The large number of analyzed virions confirmed an important heterogeneity in
159 diameter that ranges from 65 to 135 nm, unimodally and near-symmetrically distributed around
160 an average of ~100 nm (Figure 1B). From the diameter, the estimated volume of the spherical
161 TSWV particles ranges from 1.44×10^{-10} to 1.29×10^{-9} nanoliters, thus an impressive volume
162 difference close to one order of magnitude.

163 When observing semi-thin sections of TSWV viral particles fixed and embedded into a classical
164 electron microscopy resin (Figure 1C), not only did we consistently observe the heterogeneity of
165 their diameter but, interestingly, also of their inner densities which most likely represent
166 ribonucleoprotein (RNP) segments (also see Supplemental Figure S1 and Supplemental Movie S1).
167 Tomographic analysis allowed the 3D reconstruction of the volumes corresponding to these inner
168 densities (Figure 1C3 and 1C4). Remarkably, virus particles could contain 1, 2, 3 or 4 discrete
169 volumes. We relegate arguments on whether each volume represents a distinct segment or not
170 to the Discussion section.

171 Summing up all volumes of the inner density within a given virus particle provides an estimate of
172 the amount of packaged RNPs. We extracted tomograms from 25 randomly chosen particles with
173 the only constraint that they are entirely embedded in the resin (not cut through by the section)
174 and plotted their diameter against the calculated total inner density volume. Particle size and the
175 amount of encapsidated genetic material are not correlated (Figure 1 D).

176

177 **Direct Nanopore RNA sequencing reveals the packaging of both viral and complementary** 178 **strands for the three genomic segments but not of messenger RNAs**

179 After demonstrating the huge variation in the volume of packaged genetic material within
180 individual virions (four to fivefold variation in Figure 1D), we further explored the potential
181 heterogeneity in the identity/nature of RNAs. In other words, which viral RNA species (identity
182 and polarity of viral segments, viral mRNA) could be packaged in virions and in what proportion?
183 We purified virus particles from a single infected plant and extracted the viral RNA. Direct
184 Nanopore RNA sequencing was performed as indicated in the Methods section and all viral
185 reads, either of viral or complementary polarity, were mapped to the sequence of S, M and L
186 segments of TSWV isolate MR-01 (Figure 2A). According to this technology (23, 24), sequencing
187 is always initiated at the 3' extremity of the RNA and proceeds towards the 5' extremity with an
188 efficiency that decreases with length, *i.e.* the reads can be stopped for many uncontrolled
189 reasons at any time and so regions lying further from the 3' end have less chance to be reached
190 in a given read. We further calculated the sequencing depth that illustrates the number of times
191 a given nucleotide of the RNAs has been read in an experiment, and doing so we could obtain a
192 quantitative estimate as described in the Methods and as further considered in the Discussion

193 section. The trends within this sample and with this technique were: i) when summing both
194 polarities RNA-L appeared as the least accumulated (~1200 copies for which the sequencing was
195 initiated), RNA-M was slightly more frequent (~2200 copies) and RNA-S clearly dominated
196 (~11,000 copies); ii) contrary to the canonical view outlined in the Introduction, the viral
197 complementary polarity can be highly represented in packaged RNA. While the vcRNA-L is rare
198 and appears here as 3% of total segment L, the vcRNA-S accounts for 10% of segment S and
199 vcRNA-M is even more frequent than the so-called viral strand (52 vs 48%, respectively).

200 An important feature of this Direct Nanopore RNA Sequencing approach is its potential to detect
201 the presence of mRNA in purified virions. Indeed, because TSWV mRNAs acquire their cap
202 through a cap-snatching process that adds 12-20 nucleotides snatched from host mRNAs (25,
203 26), it is possible to scrutinize all available 5' sequences of the viral RNA extracted from purified
204 virions and check for the presence of a stretch of 5'-terminal non-viral (host) nucleotides. Recent
205 detailed studies of Direct Nanopore RNA sequencing reported a loss of the processive control of
206 motor proteins when the end of the RNA molecule is released from the pore, resulting in an
207 arrest of the sequencing process 11 nucleotides from the 5' extremity (23, 27, 28). We looked at
208 all reads reaching the 5' region of the TSWV RNAs for all segments and polarities. The number of
209 reads per nucleotide position crashes from about 1200 to 14 between nucleotide position 15
210 and 10 from the 5' extremity (Figure 2B), indicating an absence of cap-snatching related
211 extension expected in viral mRNAs. Moreover, among the 14 reads that extended beyond the
212 5' extremity of the viral RNAs, none could be interpreted as a cap-snatched sequence. The first
213 reason is that all but one of these 5'-extensions were much too long, between 56 and 1035
214 bases; the remaining one was only 6 nucleotides long. The second reason is that, when blasting
215 solely the first 30 nucleotides of these 14 extensions (Figure 2C), we could not find a single
216 significant match which could indicate the presence of a short sequence snatched from a host
217 mRNA (BLASTn searches against Viridiplantae or Solanaceae accessions). Our conclusion is thus
218 that the TSWV virus particles do not package even minute amounts of viral mRNA.

219 **Strand-specific RT-qPCR confirms the presence of viral and complementary strands of the**
220 **three segments each at a specific frequency in a population of TSWV particles**

221 As further debated in the Discussion section, the quantitative accuracy of Direct Nanopore RNA
222 sequencing is not established, and thus we had to confirm our conclusion with an unrelated and
223 accurate quantitative technology. We developed and thoroughly validated a strand-specific RT-
224 qPCR approach (see Methods section) and first applied it to the viral RNA sample used for Direct
225 Nanopore RNA Sequencing for comparison (Figure 3A). The result that is strikingly consistent
226 across the two techniques is the presence of the viral complementary strands for all three
227 segments, with the lowest proportion for L (~10% of total L), followed by S (~30% of total S) and
228 an astonishing near 60% of total M. Although the general trend is the same, it is noticeable, that
229 the frequency estimates of the distinct viral RNA species differ between Direct Nanopore RNA
230 Sequencing and RT-qPCR. This specific technical point will be addressed in the Discussion
231 section.

232 The presence of large proportions of viral complementary strands was a surprise. We
233 ascertained that the RNA extracted from virus particles with our protocol does not contain
234 considerable amounts of “free” RNA in solution or RNA externally associated to viral
235 membranes. We repeated the purification of virus particles from another infected plant and
236 treated half of it with RNase prior to RT-qPCR, in order to eliminate all RNA not protected in
237 virus particles. The estimates of the relative proportions of all 6 viral RNA species obtained
238 before and after this treatment are quasi-identical (Figure 3B), confirming that the viral
239 complementary strand of all three segments is indeed packaged. The total amount of RNA was
240 however drastically reduced by this treatment (four times less RNA after treatment), suggesting
241 that the RNase can also degrade packaged RNAs though likely at a slower pace. We repeated
242 this experiment after adding *in vitro* transcripts of a region of the vRNA-M, encompassing the
243 amplified region, to the purified virus suspension. The added amount of soluble RNA should
244 increase the vRNA-M copy number 84 times. This artificially enlarged proportion of vRNA-M
245 totally disappeared after RNase treatment confirming that the additional free RNA has been
246 completely degraded. In this experimental repeat, the total amount of all packaged RNA species
247 was also decreased to nearly a third, again showing that the RNA packaged into virions can be
248 degraded by prolonged RNase treatments, but much more slowly than free RNA. Of note is the
249 fact that the degradation of packaged RNA by the RNase treatment is similarly acting on all RNA
250 species, as their relative proportion remains constant (Figure 3B).

251 **The viral and complementary strands of the three TSWV segments are all packaged non-**
252 **specifically**

253 When comparing the two preparations of purified virus particles in Figure 3A and 3B, each from
254 a distinct infected plant, it is noticeable that the relative frequencies of the six viral RNA species
255 (designated hereafter as the genome formula) are alike, though some slight differences are
256 visible. This observation immediately calls for two questions. Is the genome formula
257 reproducible across virus populations from distinct individual host plants? And if yes, does it
258 stem from deterministic selective/specific packaging mechanisms or does it simply reflect the
259 amount of each of the six viral RNA species accumulated in plant tissues during the infection
260 cycle? To answer these questions, we ground one infected leaf from each of 24 host plants
261 infected in parallel. The resulting 24 crude extracts were then each split in two parts, one for
262 total RNA extraction and the other for virus particles purification followed by RNA extraction. All
263 samples were then submitted to our strand-specific RT-qPCR protocol and the genome formula
264 in total infected plant tissues and in purified virions extracted thereof were compared (Figure 4).

265 In total plant extracts, when summing up the number of copies of a given segment for both viral
266 and complementary strands, we obtained an L/M/S ratio of approximately 1/1/3. This overall
267 segment ratio can be compared with most semi-quantitative estimates found in the literature
268 for other bunyaviruses (for example see Refs(21)(11–13, 17)), where the viral and
269 complementary RNA strands were not distinguished. In contrast, accurately quantifying the two
270 polarities for each segment is more complex and such a comprehensive genome formula,
271 established in a large set of host individuals infected in parallel, has not been reported before
272 for any species in the order *Bunyavirales*.

273 In extracts from purified virus, the total number of copies of all 6 viral RNA species was 72 times
274 lower than in leaf extracts. This difference may in part stem from a loss of virions during the
275 purification process, but it also suggests that high amounts of viral RNA may exist in the host
276 plant in a non-packaged form. The genome formula of viral RNAs packaged in virion populations
277 appears strikingly similar to that of total RNA in infected plant tissues. For one out of the six RNA
278 species (for vM), the relative frequency estimated from total plant extracts and from purified
279 virion preparations is not statistically different. For the other five RNA species (vL, vCL, vCM, vS

280 and vcS) differences are statistically significant but always of very a small magnitude, still
281 consistent with the genome formula being similar in total plant extracts and in purified virions.

282 Another way to compare the proportion of the viral RNA species in infected cells and in purified
283 virus particles is to test for a possible correlation between the relative frequency of a given viral
284 RNA species in the 24 plant extracts and in the corresponding 24 populations of virions.

285 Remarkably, a positive and statistically significant correlation was revealed in 3 out of 6 cases:
286 vL, vS and vcS. The linear regression equation and the results of the tests for these three RNAs
287 are: **vL**: $\text{Freq_vL}(\text{particles}) = 0.0958832 + 0.7074039 * \text{Freq_vL}(\text{plants})$, $R^2=0.28$, test on slope: t-
288 ratio=2.91, $\text{Prob}>|t|=0.0081$; **vS**: $\text{Freq_vS}(\text{particles}) = 0.2432724 + 0.4215708 * \text{Freq_vS}(\text{plants})$,
289 $R^2=0.27$, test on slope: t-ratio=2.84, $\text{Prob}>|t|=0.0095$; **vcS**: $\text{Freq_vcS}(\text{particles}) = 0.0194624 +$
290 $0.4119993 * \text{Freq_vcS}(\text{plants})$, $R^2=0.84$, test on slope: t-ratio=10.68, $\text{Prob}>|t|<0.0001$).

291 Together, these results indicate that the viral and complementary strands of each of the three
292 TSWV genome segments are accumulated at specific and reproducible levels during the
293 infection of a given host plant species, and that they are packaged non-specifically preserving a
294 similar frequency pattern at the level of the viral population.

295

296 **Discussion**

297

298 As detailed in the introduction, numerous previous reports scattered across multiple virus
299 species in the order *Bunyavirales* have provided evidence or hinted at the possibility that virions
300 in this group often deviate from the canonical view by not packaging exactly one copy of each
301 genomic segment, and further that viral complementary strands may be packaged. How often
302 and to what extent the real situations deviate from this canonical view, and how this could
303 affect the understanding of major aspects of the biology of bunyaviruses remained largely
304 unclear. Earlier studies consistently revealed an important heterogeneity of the genetic material
305 contained in individual virions. Hence, it is unclear what is to be considered as the genome of
306 these viruses and whether it should be defined at the virion level or, more meaningfully, at a
307 larger scale. Using TSWV as a model species, we have confirmed and formally quantified the
308 stunning heterogeneity of virus particles and characterized the RNA species that can be
309 packaged and in what proportions. In addition, we have demonstrated that the genetic

310 heterogeneity at the virion level is structured in a reproducible manner at the population level,
311 extending the concept of genome formula, developed to better describe the biology of
312 multipartite viruses, to the TSWV and perhaps beyond, to the *Bunyavirales* large group of
313 segmented viruses.

314 The size distribution of viral particles has been earlier estimated for two other bunyaviruses,
315 LACV (9) and UUKV (10). The amplitude of the size variation is comparable in all three
316 bunyaviruses (including TSWV studied here) and higher than that of other segmented viruses
317 which were shown to possess specific packaging mechanisms of their genome segments, such as
318 for example Influenza virus A (29) and rotaviruses (30). Notably the size variation estimated here
319 for TSWV is even more important than that of birnaviruses (31) which have been shown to
320 encapsidate their two genomic segments in a non-specific manner, often packaging more than
321 one copy of each (32). The size distribution appeared unimodal in previous studies on
322 bunyaviruses and, in one case, was qualified as “normal”(10). However, the number of particles
323 used for these estimates was relatively low, respectively 350 and 165, and the corresponding
324 graphs seemed to reveal more particles that were smaller than the modal value. In our
325 experiment, the size distribution of 4861 TSWV particles follows a near-symmetrical distribution,
326 indicating that the number of smaller particles is similar to larger ones, and that both become
327 equally infrequent as the distance to the modal value increases (Figure 1B). Intriguingly our
328 results do not support earlier speculation that virion size variation correlates with the amount of
329 RNA content (9). We observed remarkable variability in the quantity of packaged RNA (volume
330 of electron density within virions), with some particles containing a volume of genetic material
331 four times larger than others, even among particles with the smallest diameters between 70 and
332 75 nm (Figure 1D). This lack of correlation between particle size and RNA content is consistent
333 with the observation that particles of UUKV can form without encapsidating any genetic
334 material (22, 33). A comparison of the size distribution of empty particles may reveal that
335 diameter and volume of virions are determined by the sole physical properties of the association
336 between membranes and glycoproteins. It is also possible that the packaging of the viral genetic
337 material depends on the amount of RNA that is locally and temporarily available. In any case,
338 this observation further extends the heterogeneity of packaged RNA, as this heterogeneity is
339 also large among particles of the same size class. In some of the observed viral particles, the

340 tomographic analysis reveals poorly resolved distinct inner density volumes within a single
341 virion, each potentially corresponding to a distinct segment. We observed from one to four such
342 distinct volumes per virion, consistent with the recent demonstration that particles of RVFV can
343 package a very variable number of segments(15, 17). In contrast, we did not observe any
344 particle with a complete absence of inner density, suggesting that empty particles are very rare
345 in TSWV and in our experimental set up while they appear very frequent in RVFV (15). One
346 important caution is that, due to low resolution of our tomographic analysis, the assignment of
347 each distinct inner density volume to one individual segment may not be totally reliable.
348 Heterogeneous local compactions (34, 35) and the extreme flexibility (35) of a single segment
349 may sometimes appear as more than one distinct volume upon tomography 3D reconstruction,
350 and two closely packed/entangled segments may appear as a single larger volume. Higher
351 resolution cryo-EM tomography will be required to solve the question of the spatial
352 arrangement of the segments and to reveal the range of the identity and number of segments
353 potentially co-packaged in individual virions.

354 The packaging of viral complementary sense RNA has been reported for segment S of UUKV
355 (18), for segment M of LACV (36), for segments M and S of TSWV (13), and more recently for
356 segments S, M and L of RVFV (19)(15). In these papers, the possible biological impacts of
357 packaging the two polarities of a segment have been discussed, the most obvious one being a
358 putative benefit of early expression of some of the genes due to immediate availability of both
359 strands. This would concern primarily ambisense segments, where the ratio of vRNA versus
360 vcRNA may make one or the other of the two genes encoded in opposite orientation expressed
361 early or late (18, 19). It seems less evident, however, for negative sense RNA segments for which
362 the vcRNA strand (which is not capped) cannot be translated before *de novo* transcription from
363 the vRNA where cap-snatching occurs (thus cannot be translated earlier than vRNA). Consistent
364 with this argument, the vcRNA of the negative sense segment L of TSWV could not be detected
365 within virions in earlier studies (13). In contrast, the vcRNA of the negative sense segment M of
366 LACV was detected (36), leading the authors to conclude on a possible artifactual
367 misincorporation. Going beyond detection, some of these studies provided “semi quantitative”
368 estimates. For example, Kormelink and colleagues clearly mentioned that the M segment was
369 the one with the highest amount of packaged vcRNA for TSWV, though still largely lower than

370 the vRNA. Here with two distinct technologies, Direct Nanopore RNA Sequencing and strand-
371 specific RT-qPCR, we establish that TSWV packages both polarities of its three segments L, M
372 and S. The quantification of all 6 RNA species by these two techniques reveals very consistent
373 trends, with the abundance of packaged vcRNA being $M > S > L$. However, the exact relative
374 copy number varies considerably depending on the technique and this is further discussed in the
375 next paragraph. Of note here is the general consensus that mRNAs of bunyaviruses are not
376 significantly packaged into virions because they lack the sequence driving their interaction with
377 (and coating by) the nucleoprotein (37)(19)(13, 15, 18). However, with the techniques used
378 earlier for mRNA detection within purified virus preparations, minute amounts of subgenomic
379 mRNA might have been overlooked and hence this was still considered a possibility (15). Here
380 we used the direct RNAseq approach to examine the 5' extremity of the packaged viral RNAs, in
381 search for snatched host sequences. Out of >1200 RNA 5'-extremities analyzed, not even a
382 single mRNA molecule could be identified, confirming that TSWV does not package mRNAs, at
383 least not at a level that could be considered as biologically significant.

384 Because direct RNAseq and strand-specific RT-qPCR did not reveal the same proportion of vRNA
385 versus vcRNA, particularly for segments L and S, we set up an experiment intended to control
386 the accuracy of the strand-specific RT-qPCR (Method section and Figure 3A). Conceptually, it is
387 very simple and consists of producing a solution containing a mix of the 6 RNA species from *in*
388 *vitro* transcripts in a known ratio, here 1/1/1/1/1/1, and submit it to the RT-qPCR. The resulting
389 estimates were close to the expected relative frequency of 16.6% for each of the six RNAs, the
390 largest deviation being an observed frequency of 20 % (Figure 3A grey horizontal band). We
391 believe these deviations are small insofar as they may arise because the ratios in the initial
392 control mix were not exactly equal. Indeed, quantifying transcripts in solution by spectroscopic
393 methods is poorly accurate due to variable sequence-related secondary structures engendering
394 highly variable proportions of single or double stranded regions. For this reason, and because
395 the quantitative power of the Direct Nanopore RNA Sequencing is not established (24, 27, 28), in
396 this and future work we consider the strand-specific RT-qPCR approach as the more accurate
397 and reliable.

398 In any case, as revealed with both RNAseq and RT-qPCR, the vcRNA-M is extremely frequent,
399 even more frequent than the vRNA-M. According to RT-qPCR estimates, vcRNA-S and vcRNA-L

400 respectively represent 30% and 8% of the total corresponding segment RNAs. With such high
401 amounts of packaged vcRNAs, our results again question what should be considered the viral
402 genome, v- or vc-strand. More interestingly, they also render unlikely the possibility that
403 packaged vcRNAs are biologically irrelevant. Beyond previous speculations, on a regulatory role
404 for gene expression kinetics at the early stages of infection(13, 18, 19), numerous additional
405 questions are raised: can v and vcRNA of a same segment be co-packaged in the same virion or
406 are they necessarily separated in distinct particles and only together at the population level?
407 Does a virion with the vRNA of a given segment and another virion with the vcRNA of the same
408 segment have similar infectivity? Does this also depend on the polarity of the other segments in
409 the same virion? Do the putative distinct properties of individual virions make any sense at the
410 population level through complementation and/or cooperation? Our findings provide a
411 foundation opening important issues for future research on the order *Bunyavirales*, beyond
412 TSWV and the family *Tospoviridae*.

413 Among the above non-exhaustive list of possible questions, those that appear particularly novel
414 are at the population level. If v- versus vcRNA segment ratios have a functional role, this can
415 only be conceived at the population level. It is indisputable that the RNA content of individual
416 virions is highly heterogeneous in RVFV (15, 17) and as indicated here in TSWV. We therefore
417 made use of the TSWV system to investigate the genetic composition at the population level.
418 First, in all plants of one species, so in one given environment, TSWV populations produce a very
419 similar frequency pattern for the six viral RNA species. This situation is remarkably reminiscent
420 of the genome formula described for multipartite viruses (38–40), where distinct parts of the
421 genetic information are packaged separately in distinct virus particles, but very reproducibly
422 accumulate at the population level. While a phenomenon such as the genome formula with
423 unequal ratios could be anticipated for other bunyaviruses within infected cells with
424 unpackaged genomic segments (14, 41), the situation was less predictable in purified virions as
425 it depends on whether the packaging process constrains these ratios. Some earlier studies
426 reported comparable segment frequency patterns in infected tissues and in purified virions(13,
427 15, 17), whereas others noted a variability of this pattern depending on the virus particle
428 preparation (14, 18). To resolve this issue in TSWV, we used a large set of infected plant
429 replicates to compare the genome formula in infected leaves and in virions from these leaves.

430 Beyond revealing very similar formulas, more convincing was the demonstration that the small
431 variations of a viral RNA frequency observed across the leaf extracts correlate with those
432 observed across the corresponding virus preparations. Despite an unavoidable experimental
433 “noise” induced by purification steps, reverse transcription and qPCR, such correlations could be
434 detected for three out of the six analyzed RNA species (vL, vS and vC). This experimental noise and
435 the low variations of the less frequent segments/polarities may explain that correlation was not
436 detected in the three other cases. These unique observations empirically confirm that the viral
437 RNA species accumulating during the infection cycle within a host are packaged according to
438 their frequency, thus with no apparent specific mechanisms that would bias the ratios.

439 In the same view as that currently developing for multipartite viruses(40, 42), one could imagine
440 that bunyaviruses can have emerging properties at the population level (all virions being
441 different) that would be driven by the genome formula. Whether the genome formula controls
442 gene expression (14, 39), whether it is adjustable depending on the environment (15, 43)
443 (including within insect thrips vectors), whether virions with distinct and incomplete genetic
444 information can be transmitted cell-to-cell and host-to-host separately (44–46) , and most of all
445 whether they can proficiently complement in natural situations, are all new research horizons
446 for the biology of bunyaviruses.

447

448 **Materials and Methods**

449

450 *Plants, growth conditions, inoculation, and sampling*

451 All plants were seeded and maintained in a growth chamber with a 16/8 hours day/night
452 photoperiod, a constant temperature of 20 °C, and 70% hygrometry. The tomato spotted wilt
453 virus isolate used in this study is the TSWV MR-01 (47), maintained as a -80°C frozen stock of
454 *Datura stramonium* (L.) leaves collected from plants initially infected through transmission by
455 the natural thrips vector *Frankliniella occidentalis* (Pergande). *Nicotiana rustica* (L.) plantlets at
456 the sixth leaf stage were mechanically inoculated on their fourth leaf, by rubbing a virus
457 suspension prepared from the frozen stock with 1 g of infected leaves ground in 3 ml of 0.01 M
458 phosphate buffer (pH 7.5) with the addition of Carborundum or Celite abrasive powder. Three

459 weeks post-inoculation, 1 gram of the sixth leaf of each systemically infected plant was collected
460 for further RNA or virus particle purification.

461

462 *Total RNA extraction and virus particle mini-prep protocol*

463 One gram of infected leaf tissue was homogenized for 20 seconds with 4 ceramic beads in 3 ml
464 EB (Extraction Buffer: 0.1 M sodium phosphate, pH 7, containing 0.01 M Na₂SO₃) in a 15 ml
465 Falcon tube, using the FastPrep MP grinding machine. Of these crude extracts, 200 µl could be
466 used for total RNA extraction using the Monarch Total RNA Miniprep kit (NEB #T2010, final
467 elution in 50 µl RNase free water and storage at -80°C) according to manufacturer instructions,
468 and the rest for further virus purification as described hereafter. Crude extracts were filtered
469 through a Luer Terumo syringe stuffed with Miracloth and centrifuged at 4°C and 10,000g for 15
470 minutes. The supernatant was discarded, and the pellet was rinsed with RB buffer (Rinse Buffer:
471 0.01 M sodium phosphate buffer, 0.01 M Na₂SO₃, pH 7) and rapidly resuspended in 2 ml RB
472 using a tissue grinder. After 15 minutes of 8,000 g clarification at 4°C, the supernatant was
473 collected and passed through a 5 ml Terumo Syringe containing a ClearLine Syringe 30mm AC
474 0.2 µm filter. Two to 2.5 ml of the filtrate were then overlaid on a 10-40% sucrose gradient,
475 prepared in an Ultra Clear Tube for the SW41 rotor, and centrifuged at 70,000 g for 1 hour at
476 4°C in a Beckman SW41 rotor. The opalescent band appearing in the gradient was collected,
477 diluted with RB 1/12 in a 13 ml polycarbonate tube, and pelleted for 1 hour at 70,000 g and 4°C.
478 The pelleted virions were finally resuspended in 150 µl sterile double-distilled water. When
479 larger amounts of purified virions were necessary, several samples could be pooled together in
480 the same 15ml falcon tube after the Miracloth first filtration step. In some instances, viral RNA
481 was extracted from purified virions exactly as from plant crude extracts.

482

483 *Cryo-electron microscopy and estimation of the diameter of virus particles*

484 Three microliters of freshly purified TSWV particles were overlaid onto a glow discharged
485 Quantifoil R 2/2 grid covered with a 2–3 nm ultra-thin carbon layer (Quantifoil Micro tools GmbH,
486 Germany), blotted for one second to eliminate the extra-volume, and then flash frozen in liquid
487 ethane using a CP3 cryo-plunge (Gatan inc.). Before freezing, the humidity rate was stabilized at
488 about 95 %. Cryo-EM was carried out on a JEOL 2200FS FEG microscope operating at 200 kV under

489 low-dose conditions (total dose of 20 electrons/Å²), in the zero-energy-loss mode with a slit width
490 of 20 eV. Images were taken at a nominal magnification of 50,000X, corresponding to a calibrated
491 magnification of 45,591X with defocus ranging from 1.5 to 3.0 μm, using a 4k x 4k slow scan CCD
492 camera (Gatan, inc.). The diameter estimation of TSWV particles was performed using ImageJ
493 (48).

494

495 *Electron tomography and segmentation*

496 Three hundred μL of purified virus particles suspension were fixed by addition of glutaraldehyde
497 (5% final concentration) and incubated for 45 min at 4°C. Fixed virions were then diluted in 8 ml
498 of RB and ultracentrifuged at 31,000 g for 1 hour at 4°C to eliminate the fixing agent. The pellet
499 was resuspended in 50 μl RB and added with a small volume of Chinese ink to dark stain the
500 sample. One hundred μl of 3% low melting agarose (35°C) were added and homogenized before
501 cooling overnight at 4°C. The jellified dark block was then extracted from the tube and post-
502 fixed for 1 hour at 4°C with 1% osmium tetroxide, rinsed three times in 0.1 M Cacodylate buffer,
503 and dehydrated in a series of acetone solutions as follows: two 10-minutes bathes of each of 50,
504 70, 90, 100% acetone at 4°C. The dehydrated agarose block was imbedded at room temperature
505 in 25% TAAB resin in acetone for 3 hours, in 50% TAAB resin in acetone overnight, in pure resin
506 for at least three hours, and transferred in silicon molds filled with resin. The resin block was
507 finally polymerized one night at 56°C. Thin sections 200nm thickness were prepared using an
508 ultramicrotome (LKB ultratome NOVA), and contrasted for 20 minutes in 2% Uranyl Acetate
509 followed by 3 minutes in 2% Lead Citrate.

510 Series of tilted images from semi-thin sections of embedded TSWV particles were collected on a
511 JEOL 1400 Flash electron microscope, operating at 120 kV at a magnification of 15,000X, using a
512 Oneview camera (Gatan, inc.) with defocus values ranging from -3 μm to -5 μm. The acquisition
513 was performed semi-automatically using SerialEM(49). Tilt series consisting of about 60 images
514 were collected by tilting the specimen between -60° and 60°, imaging at 2° increments, with a
515 total dose of about 700 electrons/Å². Image alignment and three-dimensional reconstructions
516 were performed using Etomo from IMOD package(50). Segmentations of particles were done
517 using Amira software (Thermo Fisher Scientific).

518

519 *Direct Nanopore RNA sequencing*

520 Total RNA was isolated from 600 µl of purified virions suspension using the Monarch Total RNA
521 Miniprep kit (NEB #T2010) and quantified using the Qubit 3.0 fluorometer (Life Technologies,
522 Carlsbad, CA). RNAs were poly(A) tailed using the *Escherichia coli* Poly(A) Polymerase NEB M0276
523 kit (New England Biolabs, Beverly, MA, USA), and further purified using paramagnetic beads
524 supplied by the RNAClean XP kit (Beckman Coulter, Brea, CA, USA). The Nanopore sequencing
525 library was constructed using the Direct RNA Sequencing Kit (SQK-RNA002), following
526 manufacturer's instructions. Direct sequencing of the poly(A) tailed RNAs was then performed on
527 one R9.4.1 Flongle flow cell (FLO-FLG001) using MinKNOW software 22.03.5.

528 Accurate base calling was performed using Guppy (v6.1.2; Oxford Nanopore Technologies,
529 available online: <https://nanoporetech.com/>) in super accuracy mode (sup) and consensus
530 filtered for a minimum Q-score quality of 7. Adapter removal was then achieved using Porechop
531 v0.2.4 (available online: <https://github.com/rrwick/Porechop>). Quality of the reads was
532 investigated using NanoPlot v1.40.0(51). Cleaned reads were further mapped in the two
533 orientations with a High/Medium Sensitivity on the three segments of tomato spotted wilt virus
534 isolate MR-01 (segment S, accession number MG593199.1; segment M, accession number
535 MG593198.1; segment L, accession number MG593197.1) using Geneious 5.5.9 (Biomatters
536 Limited). Coverage of each base of each segment was then recovered. Finally, mapped reads were
537 filtered into two groups, one large group of reads with no additional bases at the 5'-end of the
538 viral genomic RNA, and a very small group of only 14 reads having an extension at the 5'-end of
539 the viral genomic RNA.

540 As a quantitative approach, we simply counted the number of reads that were initiated at the 3'-
541 end of each of the distinct viral RNAs. We considered that this represents the number of copies
542 of a given RNA species entering a sequencing pore, which may reflect its frequency in the
543 sequencing library. To the best of our knowledge, how distinct RNA sequences could bias this
544 quantitative estimate due to secondary structures or to other reasons is thus far undetermined.

545

546 *Strand-specific reverse transcription (RT) and quantitative real time PCR (qPCR)*

547 From the extracted viral or total RNA, we aimed at quantifying specifically the viral strand
548 (vRNA) and the viral complementary strand (vcRNA) of each of the three genomic segments S, M
549 and L, avoiding the co-quantification of viral mRNAs whenever possible. Classic RT-qPCR primer
550 design revealed an auto-priming problem. We adapted an approach developed earlier to solve a
551 similar issue with the rift valley fever virus(52). This technique involves use of a unique 20
552 nucleotide-long Tag-sequence fused at the 5' extremity of the RT primer. Because the Tag-
553 sequence is later used as one of the primer pair designed for qPCR, all cDNA generated by
554 "auto-priming" (i.e. cDNA non-specifically primed by secondary structures or by co-purifying
555 small RNAs produced in the infected host) and thus lacking this Tag-sequence will not be
556 amplified. A common Tag-sequence was used for the six specific RT primers (Supplementary
557 Table S1-A), as well as for all forward primers for subsequent qPCR, the specificity at this latter
558 step being therefore determined by the reverse qPCR primers (Supplementary Table S1-A). It is
559 important to note that the distinct primer sets for RT-qPCR target plus or minus sequence
560 strands either in the 5' or 3' halves of the ambisense M and S segments. For a given polarity, the
561 chosen region was that where the subgenomic messenger RNAs do not overlap, avoiding the co-
562 amplification of the M and S segments together with their mRNA. Unfortunately, for segment L,
563 the mRNA is full length and so our approach cannot distinguish the amplification of the vcRNA-L
564 from that of the mRNA, in total RNA extracts from infected plants.

565 Before RT, the purified viral or total RNA was diluted 1/100 with RNase free water. Four μ l of
566 this dilution was used as a template, added with the RT Tagged-primers (10 μ M final
567 concentration), and the mixture was heated at 70°C for 5 minutes to eliminate secondary
568 structures that could hinder the RT efficacy. The mixture was then chilled on ice for 5 minutes,
569 complemented with avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison,
570 WI, USA), and processed according to the manufacturer's specifications. After RT, qPCR
571 reactions (40 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s) were carried out using the
572 LightCycler FastStart DNA Master Plus SYBR green I kit (Roche, NJ, USA) in a LightCycler 480
573 thermocycler (Roche,NJ, USA), following the manufacturer's instructions. Two μ l of the RT
574 products diluted 1/10 were used as qPCR template and the primers were adjusted to a final
575 concentration of 0.4 μ M. The fluorescence data were analyzed with the LinReg qPCR program
576 (53). Relative frequencies were then calculated in each sample by dividing the estimated

577 absolute fluorescence value (NO) of a given segment by the sum of the NO of all six segments
578 (three cDNA viral sense and three cDNA viral complementary sense).
579 RT is obviously a key step in the quantification of the different segments and polarities, because
580 in contrast to the qPCR, there is no evaluation and correction for possible different efficacies on
581 different RNA sequence targets. We first verified that multiplexing the 6 RT reactions (vRNA and
582 vcRNA for segment L, M and S) in a single tube did not bias the results of subsequent qPCR on
583 the corresponding cDNAs, as compared to the 6 RTs being carried out separately
584 (Supplementary Table S1-B). This possible bias being relaxed, we then experimentally controlled
585 the accuracy of the quantification of the relative copy number of the 6 distinct viral RNA species.
586 We produced *in vitro* transcripts encompassing the 6 amplified sequences, quantified each of
587 these transcripts with a NanoDrop 2000 (RNA mode), mixed them at equimolar ratio with a total
588 RNA final concentration of 2.92 ng/ml, and estimated their relative copy number with our
589 strand-specific RT-qPCR protocol (see Results section Figure 3A and Discussion).

590

591 *Plasmids construction and in vitro transcription*

592 cDNAs covering the whole TSWV MR-01 genome sequence were synthesized from TSWV-
593 infected *Nicotiana rustica* (L.) plant leaves with random primers using iScript Reverse
594 Transcription Supermix kit (Biorad, CA, USA). From these cDNAs, PCR primers (Supplementary
595 Table S1-A) were designed to amplify about 450 nucleotides from two distinct regions of
596 genomic RNA L (nucleotide position 599-1003 and 7918-8322), RNA M (291-718 and 2844-3322)
597 and RNA S (nt 261-689 and 2106-2522), using the 2x PCR master mix (Promega, Madison, WI,
598 USA), PCR Reactions were done in a thermocycler (Eppendorf) first set at 95°C 2 min, followed
599 by 30 cycles of 95°C 30 sec, 55°C 30 sec, 72°C 30 sec, and finally 72°C 15 min. PCR products were
600 cleaned using Wizard SV Gel and PCR clean up system (Promega) and cloned into pGEM-T Easy
601 vector syst (Promega). All resulting plasmids had one of the TSWV short sequences indicated
602 above flanked on one side by SP6 promoter and on the other by T7 promoter. SP6 or T7 could
603 later be used for *in vitro* transcription depending on the orientation of the cloned TSWV
604 sequence and the desired polarity of the transcript. The orientation and integrity of each of the
605 cloned TSWV sequences was verified by sequencing.

606 Three to five µg of each of these plasmids were linearized by digestion with Sal I or Nco I (New
607 England Biolabs, MA, USA), followed by enzyme inactivation at 80°C for 20 minutes.
608 Transcriptions to generate the desired plus strand or minus strand RNAs were done using SP6
609 (Nco I digests) or T7 (Sal I digests) using Maxiscript kit (Invitrogen), according to the
610 manufacturer recommendation. The template plasmids were then eliminated by DNase1
611 (RNase free) treatment (Invitrogen, USA), and the RNA finally precipitated with 0.5 M
612 Ammonium Acetate and 70% ethanol, rinsed, and stored in water at -80°C.

613

614 *Statistical analysis*

615 The histogram of the diameter distribution of virus particles was fitted to a normal distribution
616 and the significance of the fit was assessed by the Kolmogorov-Smirnov test.

617 To test whether the frequency of the six viral RNA species (segment and polarity) differed
618 between total plant extracts and purified virus particles we performed an analysis of variance
619 using the model $\text{logit}(\text{frequency_RNA species}) = \text{treatment} * \text{RNAspecies} + 1 | \text{replicate plant}$,
620 where treatment corresponds to total plant extracts vs purified virus particles. A difference in
621 the frequencies of RNA species across treatment modalities is thus indicated by a statistically
622 significant treatment by RNA species interaction. For each RNA species the statistical
623 significance of the difference in frequency across treatment modalities was assessed by Tukey's
624 HSD test. The identity of the replicate plant is introduced in the model as a random effect to
625 account for the fact that each replicate plant yielded both total plant extracts and purified virus
626 particles which are thus statistically paired.

627 The correlation of each RNA species frequency across treatment modalities was tested using
628 linear regressions on the frequencies of the RNA species (linear regressions on the logits of the
629 frequencies yielded very similar results and are not presented).

630 These three analyses were performed using JMP 13.2.1 software (SAS Institute 2016).

631

632

633

634 **Data availability**

635 All microscopic and PCR data produced are visible in the figures and supplemental material. In
636 particular, Supplemental Table S2 contains all segments' relative frequency calculated from RT-
637 qPCR to build Figure 4. Nanopore RNA sequencing data are available at
638 <https://www.ncbi.nlm.nih.gov/sra/PRJNA1020112>.

639

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641

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650

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770

771

772 **Figures legends**

773

774 **Figure 1: Particles of TSWV are highly heterogeneous in size and RNA content**

775 A. Cryo-EM image of frozen-hydrated purified virus particles.

776 B. Histogram of the diameter distribution of virus particles, $n = 4861$. The red line illustrates the
777 best fit to a normal distribution. Although not passing the Kolmogorov-Smirnov test (p -value =
778 0.035), because of very slight over-representation of large and under-representation of small
779 values, the distribution is unimodal and approximately symmetrical around the average of 100
780 nm. This histogram results from two independent virion preparations where the diameter of
781 1964 and 2897 particles was respectively estimated.

782 C. Electron tomography and segmentation of virus particles embedded in resin and semi-thin
783 sectioned. The arrow indicates one reconstituted virus particle visible in the semi-thin section.
784 The other particles, each constituting one line of the lower panel, were chosen from other
785 regions of the semi-thin section. Micrographs labeled C1: Central section of the electron
786 tomogram reconstruction of a complete viral particle. Micrographs labeled C2, C3 and C4 are
787 different representations of the segmentation of 3D reconstructions of viral particles. C2 shows
788 the surface of reconstructed virus particles, C3 combines the central section of the viral particles
789 with the segmentation of their inner density volumes (IDV) which are isolated and slightly
790 magnified in C4. Extensive views of viral particles from the semi thin section in C are presented
791 as Supplemental Figure S1 and Supplemental Movie S1.

792 D. Analysis of the relationship between the diameter of the particles and the total volume of
793 their inner density (the latter most likely corresponding to packaged ribonucleoproteins).
794 Pearson's correlation coefficient (at the top) is low, and a linear regression is not statistically
795 significant indicating an absence of correlation.

796

797 **Figure 2: Direct RNAseq of viral RNAs extracted from purified virus particles**

798 A: Sequencing depth (scale is indicated within each individual panel) per nucleotide position for
799 L, M and S segments. The three genome segments in the viral orientation are illustrated by a
800 colored thick line under the graphs where nucleotide numbering is indicated. Sequencing reads
801 for the viral (v) and viral complementary (vc) strands are respectively shown in blue (top panels)
802 and red (bottom panels). Row data of the RNAseq are available at
803 <https://www.ncbi.nlm.nih.gov/sra/PRJNA1020112>

804

805 B: Sum of the number of reads at nucleotide positions from 50 to 0 from the 5' extremity of all
806 six RNA species shown in A. The sequencing proceeds from the 3' towards the 5' end of the
807 molecule, so from right to left on the graph. The sequencing depth per nucleotide position
808 appears stable when the sequencing progresses from position 50 to position 15 towards the 5'
809 extremity, but totally crashes between position 15 and position 10, demonstrating an arrest of
810 the sequencing process when approaching the 5' extremity.

811 C: Among the > 1200 reads reaching the 5'-terminal regions of any of the six viral RNA species
812 (B), only 14 extended beyond position 0 (extension length from 6 to 1035 bases). This panel
813 shows trimmed 30 nucleotide-long sequences extended immediately upstream of the 5' end of
814 the viral segment in these 14 cases. Note that the bottom sequence had only 6 nucleotides
815 while all others had more than 30.

816

817 **Figure 3: Relative frequency of each RNA segment and polarity packaged in purified virions**

818 A: The relative proportion of each of the viral RNA segments and polarities was estimated by
819 strand specific RT-qPCR in a purified TSWV particles preparation (the very same preparation as
820 that sequenced in Figure 2). The estimate was repeated with four distinct dilutions of the same
821 purified virions preparation: 1 - 1/10 - 1/100 - 1/1000 and the variance engendered by repeated
822 measures and/or dilutions is presented as boxplots. The grey band indicates the range of values
823 within which we estimated the relative frequency of 6 artificial *in vitro* transcripts, each
824 corresponding to the RT-qPCR targeted region of a segment-polarity, after attempting to mix
825 them at equal ratio. The respective estimate for each of the transcripts within this artificial mix
826 is indicated by a colored dot.

827 B: The relative proportion of each of the viral RNA segments and polarities was estimated in
828 another purified TSWV particles preparation, obtained from a different infected host plant,
829 either with (pink) or without (blue) RNase treatment as indicated in the Results section.

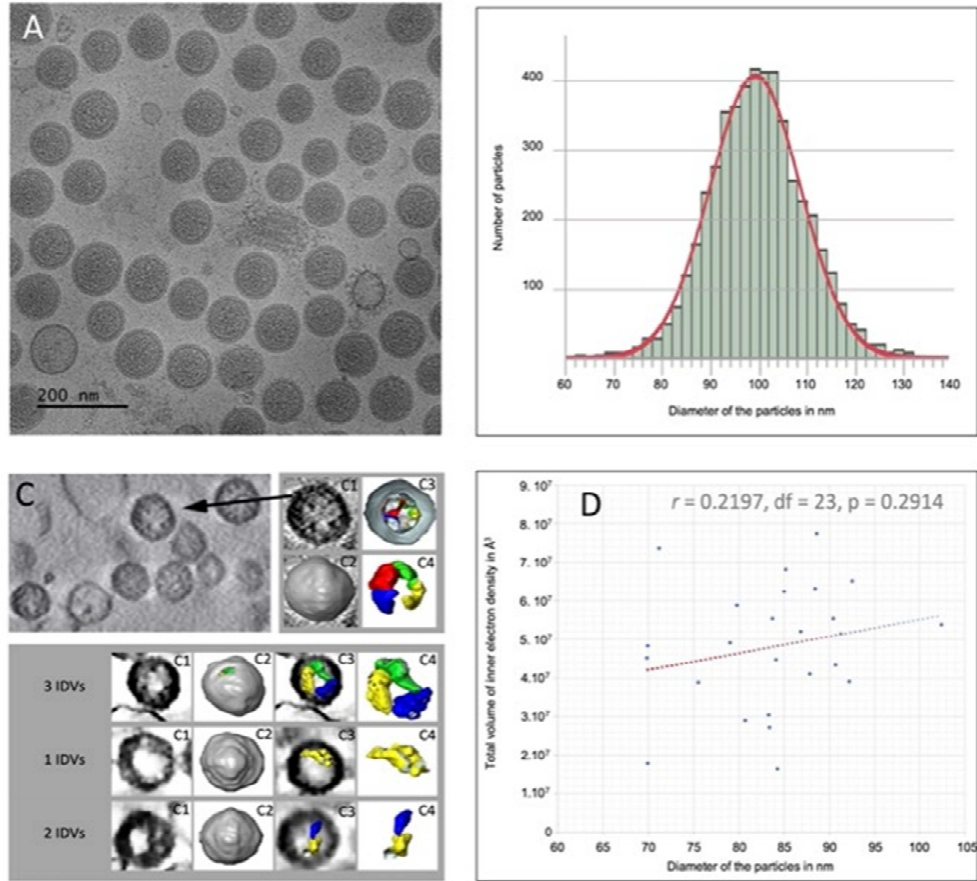
830

831 **Figure 4: Comparison of the relative frequency of each RNA segment and polarity in packaged**
832 **and total viral RNA**

833 The relative proportion of each of the viral RNA segments and polarities was estimated by
834 strand specific RT-qPCR either in total RNA extracts from infected plant leaves (Total; n = 24) or
835 in virions purified from these leaves (Virions; n = 24). The results are represented as box plots
836 with the horizontal bar indicating the median value of the distribution. The identity of the viral
837 RNA is indicated above the graph. Statistical analysis was performed as indicated in the Methods
838 section. The grey shaded panel indicates the RNA species for which the difference in frequency
839 observed in total RNA extracts and in purified virions is not statistically significant. The segments
840 frequencies calculated from RT-qPCR on these 48 samples are available in Supplemental Table
841 S2

842

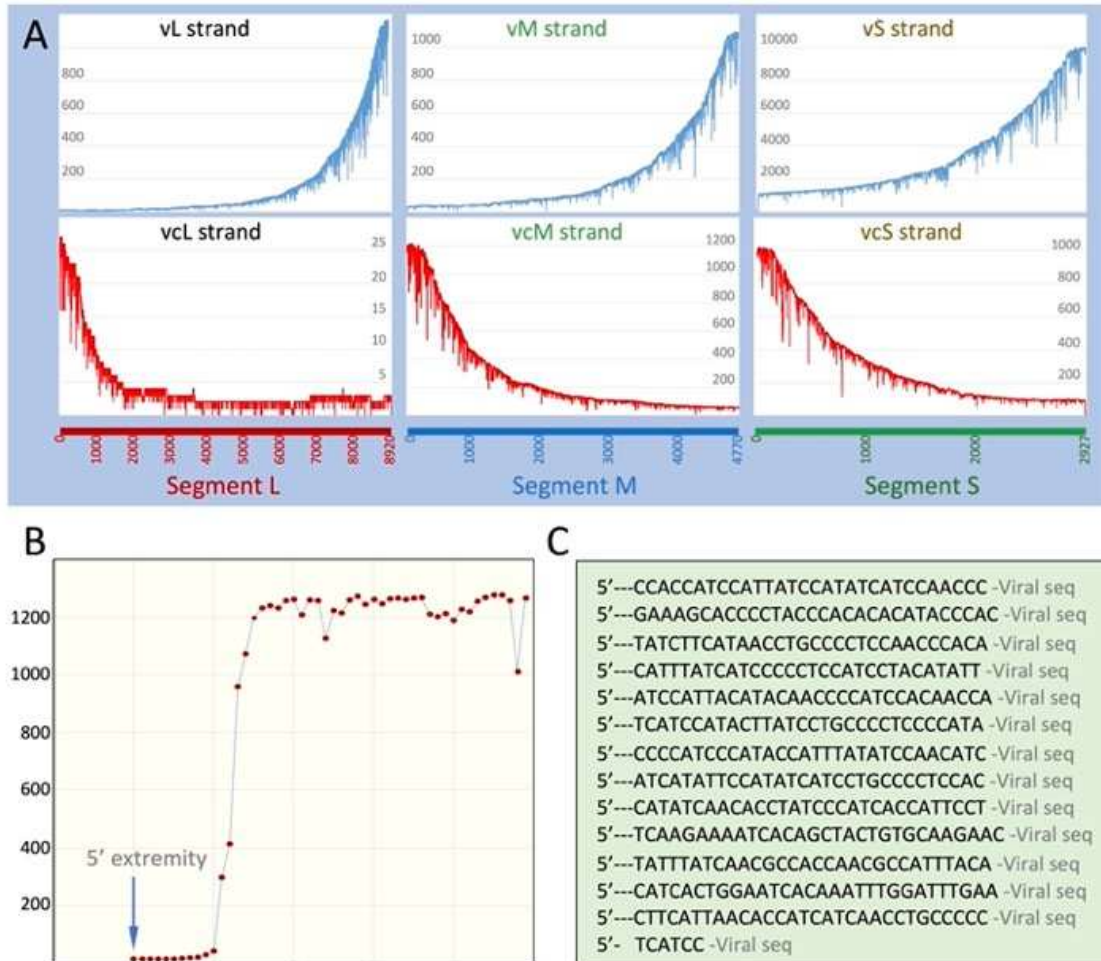
843 **Figure 1**



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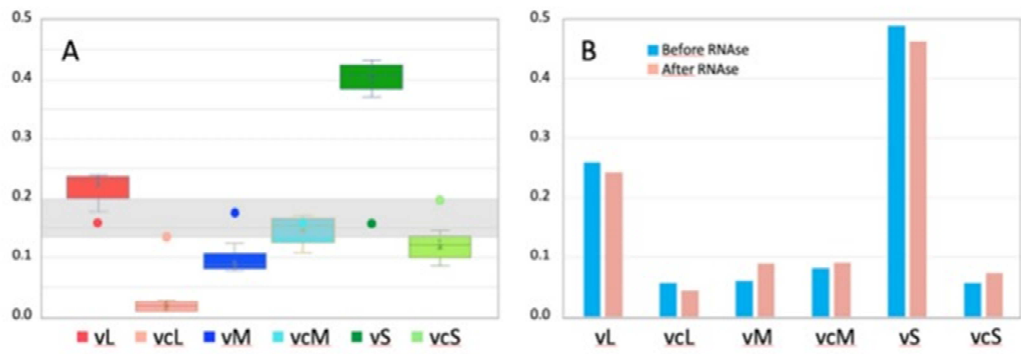
846 **Figure 2**



847

848

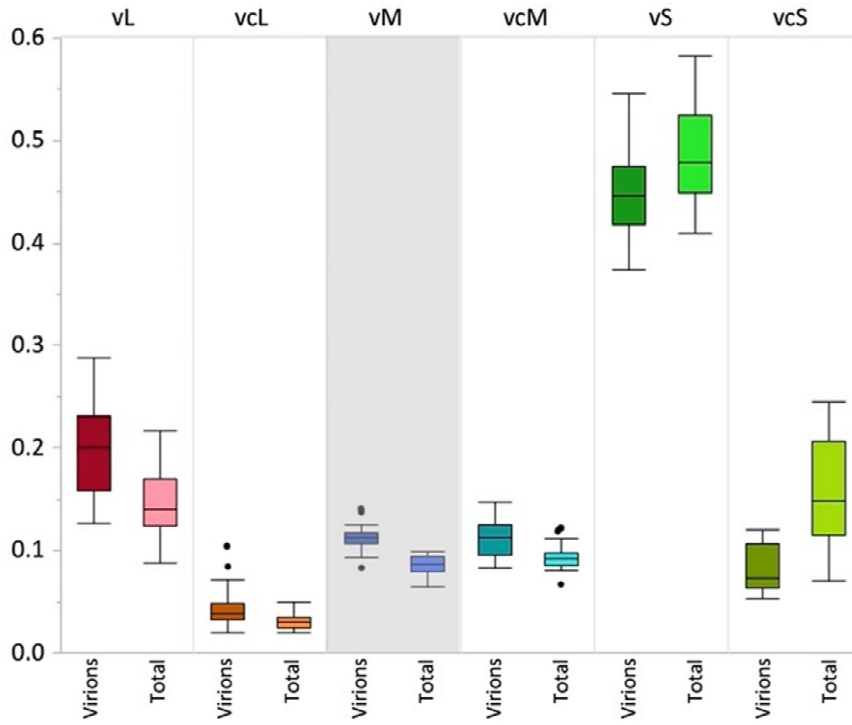
849 **Figure 3**



850

851

852 **Figure 4**



853

854

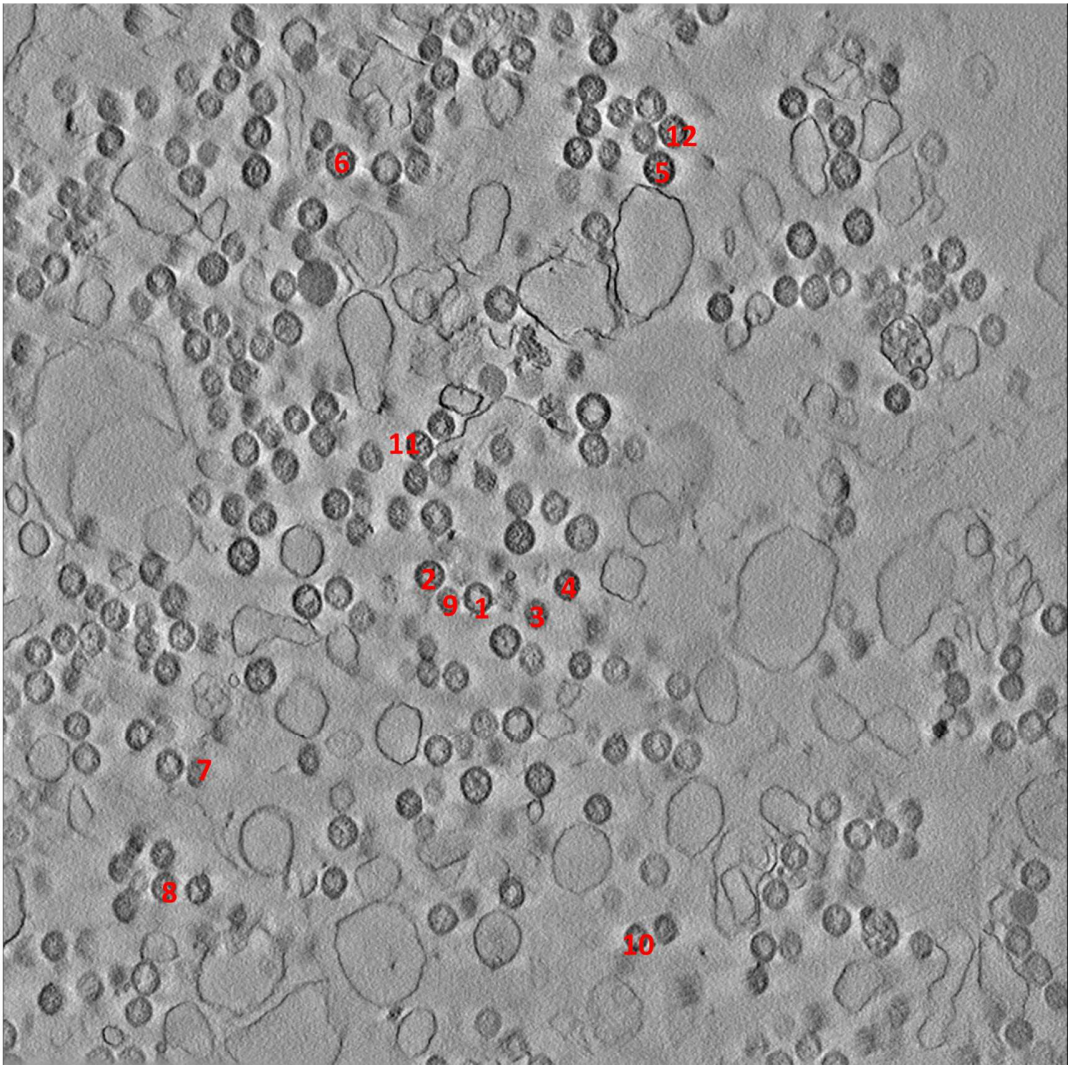


Table S1-A: Sequence of primers used for cloning, reverse transcription, PCR, and qPCR

Oligonucleotide Name	Purpose	Nucleotide sequence (5'-3')
TSWV-SRNA-F2106	cloning	GGATTGCTGGAGCCAAGTATAG
TSWV-SRNA-R2522	cloning	GATTGGAGCCACTGACATGA
TSWV-SRNA-F261	cloning	CACTGCAAAGGTAGGGAATCT
TSWV-SRNA-R689	cloning	GCTTGAACAGTACAGCCATTC
TSWV-MRNA-F2844	cloning	GCAGAATGTTCTCTGGTATGT
TSWV-MRNA-R3322	cloning	GTCTCAAATGCCATGTCTAT
TSWV-MRNA-F291	cloning	CATCTCCTTTGGAACCTATGA
TSWV-MRNA-R718	cloning	GTACTCTGGCTGCACATCAA
TSWV-LRNA-F599	cloning	CTGTGATGCCAGGACTGAA
TSWV-LRNA-R1003	cloning	TGCTGGTCCAGTGACTAA
TSWV-LRNA-F7918	cloning	TGCTGGTCCAGTGACTAA
TSWV-LRNA-R8322	cloning	CTGTGATGCCAGGACTGAA
TSWV-vS-RT-Tag	cDNA/RT	GGCCGTCAACGTGGCGCCTAA GCTTCTCACCTTTGATTC
TSWV-vcS-RT-Tag	cDNA/RT	GGCCGTCAACGTGGCGCCTAA TTTGTCTCTGCTCAGCTCC
TSWV-vM-RT-Tag	cDNA/RT	GGCCGTCAACGTGGCGCCTAA TGCGTTGCACACCATCTAT
TSWV-vcM-RT-Tag	cDNA/RT	GGCCGTCAACGTGGCGCCTAA TTTGTGGCAACGGGAAGC
TSWV-vL-RT-Tag	cDNA/RT	GGCCGTCAACGTGGCGCCTAA CTGTGGGTGGTTCCAAC
TSWV-vcL-RT-Tag	cDNA/RT	GGCCGTCAACGTGGCGCCTAA CTATTGCTGGGATGCCGAT
Tag-Forward-qPCR	qPCR	GGCCGTCAACGTGGCGCCTAA
TSWV-vS-qPCR	qPCR	ATAGCCAAGACAACACTGATC
TSWV-vcS-qPCR	qPCR	GAGCCCTTGTTCAGCTTC
TSWV-vM-qPCR	qPCR	GCCTCATACAGACCAACCGA
TSWV-vcM-qPCR	qPCR	GGGTTGGTATAGTGGGGCA
TSWV-vL-qPCR	qPCR	CCTATTGCTGGGATGCCGAT
TSWV-vcL-qPCR	qPCR	CTGTGGGTGGTTCCAAC

The non-viral Tag sequences are shown in bold face

Primers where used :

- (i) to generate PCR products that have later been cloned to produce plasmids for in vitro transcription (cloning)
- (ii) for the production of cDNAs through reverse transcription (cDNA/RT)
- (iii) for qPCR

Table S1-B: comparison of single RT and multiplexed RT

	vRNA-L	vcRNA-L	vRNA-M	vcRNA-M	vRNA-S	vcRNA-S
RT made with individual primers in individual tubes	0.31	0.29	0.07	0.09	0.21	0.04
RT made with all six primers in a single tube	0.28	0.30	0.07	0.09	0.22	0.04

The same RNA extract from infected plants was used as a template for RT-qPCR in all cases

Values represent the relative proportion of each viral RNA species in the RNA extract as estimated by RT-qPCR